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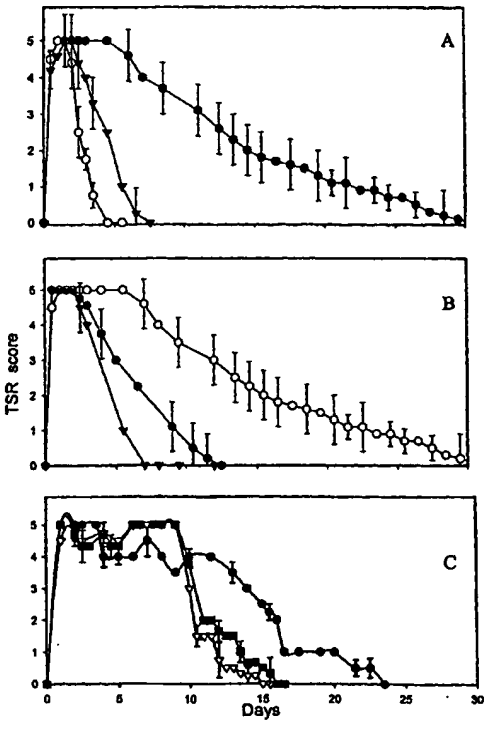
(54) Title: BONT/E OR SNAP-25E FOR TREATING BOTULINUM TOXIN A OR C1 POISONING AND INHIBITING MUSCLE CONTRACTION



(57) Abstract: A method for treating a patient with Botulinum toxin A (BoNT/A) or Botulinum toxin C1 (BoNT/C1) poisoning, wherein the patient is administered Botulinum toxin E (BoNT/E) or a polynucleotide encoding and capable of expressing BoNT/E, or a fragment derivable by cleavage of synaptosomal-associated polypeptide of 25 kDa (SNAP-25) or a variant thereof by BoNT/E ($SNAP\text{-}25_E$) or a polynucleotide encoding and capable of expressing $SNAP\text{-}25_E$. The patient may have botulism acquired naturally or accidentally, or may have been injected with BoNT/A or BoNT/C1 for medical purposes. A method for treating a patient in need of short duration inhibition of exocytosis in a cell of the patient, wherein the patient is administered BoNT/E or a polynucleotide encoding and capable of expressing BoNT/E, or a fragment derivable by cleavage of synaptosomal-associated polypeptide of 25 kDa (SNAP-25) or a variant thereof by BoNT/E ($SNAP\text{-}25_E$) or a polynucleotide encoding and capable of expressing $SNAP\text{-}25_E$ and is not administered BoNT/A, B, C, F or G. The patient may be in need of inhibition of exocytosis of less than 14 days' duration.

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BONT/E OR SNAP-25E FOR TREATING BOTULINUM TOXIN A OR C1 POISONING AND INHIBITING MUSCLE CONTRACTION

The present invention relates to botulinum toxins (BoNTs) and their use in medicine.

Botulism is a paralyzing disease caused by the toxin of *Clostridium botulinum* (see Cherington (1998) *Muscle Nerve* **21(6)**, 701-710 for a review). The toxin produces skeletal muscle paralysis by producing a presynaptic blockade to the release of acetylcholine. The several types of botulinum neurotoxin act at the nerve terminal. Since the discovery of the toxin about 100 years ago, five clinical forms of botulism have been described: 1) classic or foodborne botulism; 2) wound botulism; 3) infant botulism; 4) hidden botulism; 5) inadvertent botulism. A clinical pattern of descending weakness is characteristic of all five forms. Almost all human cases of botulism are caused by one of three serotypes (A, B, or E). Classic and wound botulism were the only two forms known until the last quarter of this century. Wound botulism was rare until the past decade. Now there are increasing numbers of cases of wound botulism in injecting drug users. Infant botulism, first described in 1976, is now the most frequently reported form. In infant botulism spores of *Clostridium botulinum* are ingested and germinate in the intestinal tract. Hidden botulism, the adult variant of infant botulism, occurs in adult patients who usually have an abnormality of the intestinal tract that allows colonization by *Clostridium botulinum*. Inadvertent botulism is the most recent form to be described. It occurs in patients who have been treated with injections of botulinum toxin for dystonic and other movement disorders.

Laboratory proof of botulism is established with the detection of toxin in the patient's serum, stool, or wound. The detection of *Clostridium botulinum* bacteria in the stool or wound should also be considered evidence of clinical botulism. Electrophysiological studies can provide presumptive evidence of botulism in patients with the clinical signs of botulism. Electrophysiological testing can be especially helpful when bioassay studies are negative. The most consistent electrophysiological abnormality is a small evoked action potential in response to a single supramaximal nerve stimulus in a clinically affected muscle. Post-tetanic facilitation can be found in some affected muscles. Single-fibre EMG studies typically reveal increased jitter and blocking, which become less marked following activation.

In humans, neuromuscular paralysis resulting from exposure to BoNT is often fatal, and survivors require a year or more to achieve full recovery [Sloop *et al* (1997) *Neurology* **49**, 189-194]. The major treatment for severe botulism is advanced medical and nursing supportive care with special attention to respiratory status.

Seven homologous serotypes of BoNT, termed A-G, are produced by different *Clostridium botulinum*; each has a molecular weight of about 150 kD and consist of a heavy and light chain (LC) linked by a disulphide bridge and non-covalent bonds. BoNTs target motor nerve endings by binding avidly to distinct ecto-acceptors, exclusively located on cholinergic presynaptic membranes, with subsequent acceptor-mediated uptake and translocation to the cytosol where they block transmitter release (Dolly *et al* (1994)). This is due to the LCs being Zn^{2+}-dependent neutral endoproteases with each having a strict specificity to cleave a distinct peptide bond, in one (usually) of three proteins essential for Ca^{2+}-regulated

transmitter release: SNAP-25, synaptosomal-associated protein of Mr=25 kD (BoNT/A, E, C1); syntaxin 1 (BoNT/Cl) and synaptobrevin (BoNT/B, D, F or G). Cleavage of SNAP-25 (Blasi *et al.*, 1993; Schiavo *et al.*, 1993), synaptobrevin (Deloye *et al.*, 1996; Schiavo *et al.*, 1994; Schiavo *et al.*, 1993; Schiavo *et al.*, 1993) or syntaxin1 (Foran *et al.*, 1996; Schiavo *et al.*, 1993) results in blockade of regulated exocytosis. Both A and E cleave SNAP-25, within the C-terminus at peptide bonds (Gln^{197}-Arg^{198} and Arg^{180}-Ile^{181}, respectively) in close proximity to each other, but strikingly induce neuromuscular paralysis of long (several weeks) and short (few days) durations (Eleopra *et al.*, 1998; see also Keller *et al* (1999) *FEBS Lett* 456, 137-142).

SNAP-25, syntaxin1 and synaptobrevin are termed SNAREs (soluble NSF-attachment protein receptor, where NSF is *N*-ethylmaleimide-sensitive fusion protein). SNAP-25 and syntaxin1 are target membrane SNAREs (tSNAREs) whereas synaptobrevin is a vesicle-membrane SNARE (vSNARE). Multiple isoforms of vSNARES and tSNARES have been described (which are reviewed briefly in, for example, Gonelle-Gispert *et al* (1999) *Biochem J* 339, 159-165 and more extensively in Linial (1997) *J Neurochem* 69, 1781-1792).

One approach to treating botulism is to develop a small, effective and specific inhibitor of the protease for each light chain (LC), which must be amenable to targeting and delivery inside the poisoned nerve terminals (see Schmidt *et al* (1998) *FEBS Lett* 435(1), 61-64). Small synthetic peptide inhibitors are described, the most avid being N-acetyl-CRATKML-carboxamide, with a K_i of ~2μM.

Although prompt administration of neutralising antibodies can reduce the possibility of death in patients with botulism, these are ineffective toward toxin already internalised within the motor nerves of patients displaying the symptoms of botulism. For persons at risk of botulism, for example military personnel, vaccination with botulinum toxoid is possible. However, there is growing concern about vaccination with toxoid, because of the widespread and successful use of BoNT/A in the treatment of numerous muscle movement disorders; vaccination with toxoid may render subsequent treatment with BoNT/A ineffective. Muscle movement disorders that may be treated using BoNT/A include a variety of dystonias and dysphonias – see, for example Gordon (1999) The role of botulinum toxin type A in treatment-with special reference to children. *Brain Dev* **21(3)**, 147-51. Increasing unpopularity of this prophylaxis may lead to its restricted application. In view of this, and the other above-noted factors, there is a need to design novel treatments for human botulinum poisoning, for example botulism, including reversal of the effects of therapeutic administration of botulinum toxin.

WO01/18038 describes methods for inhibiting SNARE-dependent exocytosis in a cell wherein a fragment, variant, chimaera (fusion; tagged) or derivative of a SNARE or a chimaera (fusion) of such a fragment, variant or derivative (inhibitory SNARE) that is capable of inhibiting SNARE-dependent exocytosis is supplied to the cell, for example in a patient. This method of inhibiting exocytosis may be useful as an alternative to the inhibition of exocytosis by the administration of a clostridial toxin to cells susceptible to such inhibition by a clostridial toxin. Alternatively, it may be useful in inhibiting exocytosis in cells that are not susceptible to clostridial toxin *in vivo*.

Methods of reversing such inhibition of exocytosis may be useful.

Therapeutic uses of botulinum toxins are also described in, for example WO95/17904 and WO94/28923. WO95/17904 suggests the use of BoNT/F when a short duration of action is required. WO9 4/28923 suggests the use of combinations of botulinum toxins in order to control the duration of therapeutic activity. There is no suggestion that the combination may have a shorter duration of therapeutic activity than that of the component toxins when administered individually.

There is a need for treatments where a significantly shorter duration of action is preferred and full and rapid recovery of function is achieved.

We demonstrate that administration of an agent that reduces the amount of an inhibitory, for example toxin-cleaved, SNARE protein in a cell, and/or alters the location of the inhibitory SNARE protein in the cell, is useful in reversing inhibition of exocytosis, for example arising from exposure of the cell to botulinum toxin. We provide methods of treatment of inhibition of exocytosis, for example methods of treating botulinum toxin poisoning. These may be used in conjunction with other treatments, for example administration of a protease inhibitor, as described above.

When treating botulism, this treatment may afford relatively fast rescue of transmitter release, alleviating the symptoms when most severe and taking the patient out of the critical state. Furthermore, this treatment may pre-empt BoNT/A-induced nerve sprouting and long-term remodelling of the motor endplates (de Paiva *et al* (1999) *Proc. Natl. Acad. Sci. (USA)* 96,

3200-3205) and may avoid the poisoning-associated extensive atrophy of the muscle fibres and negate the need for months of rehabilitation.

The methods may be useful in reversing therapeutic inhibition of exocytosis, for example when the inhibition is more severe than required, or has been generated in the wrong cells, for example in the wrong muscle group.

We also demonstrate that the impact of BoNT/E on treated cells is less prolonged than other botulinum toxins, for example BoNT/F. We provide methods of treatment which provide inhibition of exocytosis of short duration and good recovery.

A first aspect of the invention provides a method for treating a patient with Botulinum toxin A (BoNT/A) or Botulinum toxin C1 (BoNT/C1) poisoning, wherein the patient is administered Botulinum toxin E (BoNT/E).

A second aspect of the invention provides the use of BoNT/E in the manufacture of a medicament for the treatment of a patient with BoNT/A or BoNT/C1 poisoning.

A third aspect of the invention provides a method for treating a patient in need of reversal of inhibition of exocytosis in a cell of the patient caused by contact of BoNT/A or BoNT/C1 with the said cell, wherein the patient is administered BoNT/E.

A fourth aspect of the invention provides the use of BoNT/E in the manufacture of a medicament for the treatment of a patient in need of

reversal of inhibition of exocytosis in a cell of the patient caused by contact of BoNT/A or BoNT/C1 with the said cell.

BoNT/A poisoning is more prevalent than BoNT/C1 poisoning; it is therefore preferred that the toxin is BoNT/A. The BoNT/E is supplied to affected cells of the patient, as discussed further below.

Treatment with BoNT/E may be useful in treatment of BoNT/A or BoNT/C1 poisoning because it may prevent or diminish nerve cell sprouting (as described in Example 1) and the resultant, highly undesirable need for months of rehabilitation. In the absence of significant nerve cell sprouting, recovery of muscle function may to be quicker and more complete than when significant sprouting has taken place.

In relation to any of the preceding aspects of the invention, the active ingredient may alternatively be a polynucleotide encoding and capable of expressing BoNT/E, or SNAP-25$_E$ or a polynucleotide encoding and capable of expressing SNAP-25$_E$ (as discussed further below).

A fifth aspect of the invention provides the use of an agent which is capable of (1) reducing the amount of a fragment, variant, chimaera or derivative of a SNARE or a chimaera of a said fragment, variant or derivative (inhibitory SNARE) that is capable of inhibiting SNARE-dependent exocytosis in a cell in which an inhibitory SNARE is present, and/or (2) altering the location of the inhibitory SNARE in a cell in which an inhibitory SNARE is present, in the manufacture of a medicament for the treatment of a patient in need of reversal of inhibition of SNARE-dependent exocytosis in a cell in which the inhibitory SNARE is present.

A sixth aspect of the invention provides a method for reversing the inhibition of SNARE (soluble (*N*-ethylmaleimide-sensitive fusion protein)-attachment protein receptor)- dependent exocytosis in a cell in which a fragment, variant, chimaera or derivative of a SNARE or a chimaera of a said fragment, variant or derivative (inhibitory SNARE) that is capable of inhibiting SNARE-dependent exocytosis is present, the method comprising the step of supplying to the cell an agent which is capable of reducing the amount of the inhibitory SNARE in the cell and/or altering the location of the inhibitory SNARE in the cell, wherein the method is performed *in vivo*, or alternatively wherein the inhibitory SNARE is present in the cell as a result of circumstances other than exposure of the cell to BoNT/A (or preferably any clostridial, for example botulinum, toxin, for example BoNT/C1). In the latter case the method may be performed *in vivo* or *in vitro*.

The term SNARE (soluble (*N*-ethylmaleimide-sensitive fusion protein)-attachment protein receptor) is well known to those skilled in the art, for example Gonelle-Gispert *et al* (1999) *Biochem J* **339**, 159-165 and Linial (1997) *J Neurochem* **69**, 1781-1792. SNARE polypeptides are considered to be involved in Ca^{2+}-regulated exocytosis, for example release of neurotransmitters from nerve terminals, insulin (stored in large dense-core granules) release, for example from pancreatic B cells or the HIT (hamster) or RIN (rat) insulin-secreting cell lines and evoked exocytosis from chromaffin cells. Chromaffin cells are the secretory cells of the adrenal medulla.

It is preferred that the said cell is one in which it is desirable to reduce inhibition of Ca^{2+}-regulated exocytosis arising from the presence of the inhibitory SNARE in the cell. The cell may be a cell in which the inhibitory SNARE is present as a result of exposure of the cell to a clostridial toxin, for example a botulinum toxin, for example BoNT/A or BoNT/C1. Thus, the cell may be a cell in which a clostridial toxin is capable of inhibiting Ca^{2+}-regulated exocytosis. Where the clostridial toxin is a botulinum toxin, the cell may be a cholinergic neuron. Where the clostridial toxin is a tetanus toxin, the cell may be an inhibitory neuron in the spinal cord.

In an alternative embodiment, the inhibitory SNARE is present in the cell as a result of circumstances other than exposure of the cell to a clostridial toxin, for example BoNT/A or BoNT/C1. The inhibitory SNARE may be present in the cell as a result of supply of the inhibitory SNARE to the cell, for example by expression of the inhibitory SNARE in the cell from a recombinant polynucleotide or as a result of administration of the inhibitory SNARE to the cell, for example as described in WO01/18038.

The said cell (for example, cell in a patient) is of a type that is capable of performing SNARE-dependent exocytosis in the absence of the inhibitory SNARE. It may be a nerve cell (for example a cholinergic nerve cell or an inhibitory interneurone), adreno-chromaffin cell, insulin-secreting cell (for example a pancreatic B cell), endocrine cell lines of intestinal origin (for example cholecystokinin (CCK)-secreting cells, similar to cell lines STC-1 and GLUTag; see, for example Nemoz-Gaillard *et al* (1998) *FEBS Lett* **425(1)**, 66-70) or endocrine non-intestinal cell lines (similar to, for example cell lines CA-77 and HIT-T15). As noted in WO01/18038, inhibition of

exocytosis in a cholinergic nerve cell by supply of an inhibitory SNARE may be useful in producing paralysis, for example localised paralysis, in a manner similar to the use of BoNT/A for the treatment of muscular movement disorders or for cosmetic treatment, for example in which facial muscles are relaxed. This may be useful in, for example, patients that cannot be successfully treated using a clostridial toxin, for example BoNT/A, as a consequence of immunity to the clostridial toxin, for example as a result of previous exposure to the clostridial toxin, for example as a result of previous vaccination against botulism, for example vaccination using a pentavalent BoNT/A toxoid. Disorders which may be appropriate to treat, particularly in the field of pediatrics, are discussed in Gordon (1999) *Brain Dev* 21(3), 147-51 and may include strabismus and blepherospasm, spastic cerebral palsy,the extrapyramidal form of cerebral palsy, forms of dystonia, (generalized or focal), spasmodic torticollis and pain (for example back pain) caused by muscle spasms. Inhibition of exocytosis in (ie blocking of discharges from) cholinergic sympathetic and parasympathetic terminals may be beneficial, for example in the treatment of autonomic disorders, for example focal hyperhidrosis (excessive sweating), lacrimation and salivation, particularly prominent in patients suffering from Parkinson's disease.

Adreno-chromaffin cells are the secretory cells of the adrenal medulla and secrete, for example, adrenaline, the effects of which closely resemble those brought about by activity of the sympathetic nervous system. Inhibition of exocytosis from adreno-chromaffin cells may be useful in the treatment of disorders or conditions in which excessive adrenaline release may be involved, for example stress.

Delivery of BoNT/B or E/ into adipocytes blocks the SNARE-dependent fusion of glucose transporter 4-containing vesicles with the cell surface preventing the majority of insulin-stimulated glucose uptake (i.e. control of weight gain). Chen *et al.*, 1997, Biochem. 36 p5719-5728). Thus, inhibition of exocytosis in these cells may be beneficial. It may also be appropriate to block inappropriate catecholamine secretion from adrenal chromaffin cells or pheocytomyocytomas. It is likely that every cell that exhibits a regulated membrane fusion event will require SNAREs; thus, abnormalities of secretion in any such cell may be potentially treatable using an inhibitory SNARE, as described in WO01/18038.

The methods or uses according to the present invention may be useful in reversing (including partially reversing, or modulating) such inhibition, for example if the inhibition of exocytosis is no longer required, or if a reduced level and/or duration of inhibition is required.

It is preferred that the cell is a mammalian cell, more preferably a human or rodent cell, still more preferably a human cell.

Examples of SNAREs include SNAP-25 (synaptosomal-associated protein of 25 kDa), syndet (or SNAP-23), the VAMP (vesicle-associated membrane protein) vSNARE sub-family (which includes VAMP-1 (synaptobrevin1), VAMP-2 (synaptobrevin2) and cellubrevin) and the syntaxin tSNARE sub-family which has more than 12 isoforms with different tissue distributions as well as different cellular localizations. Syntaxin 1a and 1b are largely neuron or neuroendocrine specific.

It will be appreciated that a SNARE may be capable of forming a complex with the NSF (*N*-ethylmaleimide-sensitive fusion protein) or a SNAP (soluble (*N*-ethylmaleimide-sensitive fusion protein)-attachment protein). SNAREs may contain homologous domains that form coiled-structures that may mediate interaction between SNAREs, as known to those skilled in the art.

SNAP-25 is present in two isoforms (a and b) in neurons (Bark (1993) *J Mol Biol* **233**, 67-76; Bark & Wilson (1994) *Gene* **139**, 291-292). The isoforms appear to arise from alternative splicing of two divergent versions of exon 5 and differ by nine amino acids and the spacing of four cysteine residues which are palmitoylated and participate in the membrane association of SNAP-25. Both forms appear to be able to support insulin secretion in HIT cells (Gonelle-Gispert *et al* (1999) *Biochem J* **339**, 159-165). Zhao *et al* (1994) *Gene* **145(2)**, 313-314 report that human SNAP-25 may have an identical amino acid sequence to mouse SNAP-25. Human and mouse SNAP-25b are 95.6% identical at the amino acid level with a 100% homology at the relevant C-terminus. Non-identical residues are located towards the N-terminus and have analogous amino acid substitutions. There are six amino acid changes of which 5 are conservative.

SNAP-23 (also termed syndet) is a homologue of SNAP-25 that appears to be ubiquitously expressed and has approximately 60% amino acid identity with SNAP-25 (human SNAP-23: Ravichandran *et al* (1996) *J Biol Chem* **271**, 13300-13303; mouse SNAP-23: Araki *et al* (1997) *Biochem Biophys Res Comm* **234**, 257-262; Wang *et al* (1997) *J Cell Sci* **110**, 505-513). SNAP-23 appears to be able to perform the function of SNAP-25 in insulin

secretion when overexpressed (Sadoul *et al* (1997) *J Cell Biol* **128**, 1019-1028).

SNAP-25 is cleaved by BoNT/A between Gln197 and Arg198 (numbering of full length SNAP-25). It is cleaved by BoNT/C1 between Arg198 and Ala199 (numbering of full length SNAP-25) and by BoNT/E between Arg180 and Ile181 (numbering of full length SNAP-25). Human SNAP-23 does not appear to be cleaved by BoNT/A, BoNT/C1 or BoNT/E *in vitro*. Rat SNAP-23 appears to be cleaved by BoNT/E and to a limited extent by BoNT/A *in vitro*. (see, for example, Vaidyanathan *et al* (1999) *J Neurochem* **72**, 327-337 and Figure 7).

Syntaxin 1 is cleaved by BoNT/C1 and synaptobrevin (Sbr) by BoNT/B, /D, /F, /G and TeTx [reviewed by Pellizzari, R., Rossetto, O., Schiavo, G., and Montecucco, C. (1999) Tetanus and botulinum neurotoxins: mechanism of action and therapeutic uses. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **354**, 259-68]. Human VAMP-2 (synaptobrevin2) is cleaved between Gln76 and Phe77 by TeTx (see, for example Shiavo *et al* (1992) *EMBO J* **11**, 3577). Cellubrevin is also cleaved by TeTx.

Galli *et al* (1998) *Mol Biol Cell* **9(6)**, 1437-1448 reports that syntaxin 3, SNAP-23 and a tetanus neurotoxin-insensitive VAMP (TI-VAMP) are insensitive to clostridial neurotoxins. As mentioned in Gonelle-Gispert *et al* (1999), many isoforms (more than 10) of syntaxin exist. The susceptibilities of isoforms 1 - 5 to BoNT/C1 cleavage have been assessed and it appears that only 1, 2 and 3 are sensitive. In addition, within each group many minor variants have also been reported. It will be appreciated that the sensitivities of each variant to cleavage may be different.

The said inhibitory SNARE may be a fragment of SNAP-25, synaptobrevin or syntaxin1, which terms are defined above. It is preferred that it is a fragment of SNAP-25, still more preferably a fragment of SNAP-25 derivable by cleavage of SNAP-25 by BoNT/A or BoNT/C1, for example derivable by cleavage of SNAP-25 or a variant thereof by BoNT/A between residues 197 and 198 of full length SNAP-25. It will be appreciated that by "derivable" is included the meaning of intellectually derivable; thus, the fragment may be synthesised, for example using techniques of molecular biology or synthetic peptide synthesis, without need for cleavage by a clostridial toxin, for example BoNT/A. It is particularly preferred that the said inhibitory SNARE is a fragment of SNAP-25 (or a variant thereof) wherein residues corresponding to residues 198 (or less preferably 199) to 206 of full length mouse SNAP-25 are not present. Thus, the fragment may consist of residues identical to residues 1 or about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 140, 160, 170 or 180, preferably between 1 and about 140, to 197 of full length SNAP-25 or a variant thereof. Alternatively, the fragment may consist of residues identical to residues 1 or about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 140, 160, 170 or 180, preferably between 1 and about 140, to 198, 199, 200 or 201 of full length SNAP-25 or a variant thereof. Such fragments of SNAP-25 may be capable of inhibiting SNARE-dependent exocytosis, as described in WO01/81038.

It is preferred that the fragment is not one that consists of residues equivalent to or identical to residues 1 or about 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 140, to 180 of full length SNAP-25 (SNAP-25(1-180)); such a fragment may be derivable by cleavage of SNAP-25 by

BoNT/E. Such a fragment may be capable of inhibiting SNARE-dependent exocytosis (see Figure 8 and as described in WO01/18038), but is not considered to be as persistent an inhibitor of exocytosis as SNAP-25(1-197); thus, inhibition arising from a fragment derivable by cleavage of SNAP-25 by BoNT/A may be reversed/reduced by cleavage of the fragment by BoNT/E, which gives a transient (full but rapidly reversed) block.

Methods of determining that a polypeptide is capable of inhibiting SNARE-dependent exocytosis are described, for example, in WO01/18038 and in Huang *et al* (1998) *Mol Endocrinol* 12(7), 1060-1070.

Alternatively, Ferrer-Mental *et al.*, 1998 have shown that polypeptides encompassing the last 20 or 26 C terminal residues of SNAP-25, blocked evoked secretion from permeabilised chromaffin cells. As a 65-mer SNAP-25 C terminal fragment can operate in exocytosis (Chen *et al* 1999) and a polypeptide of the last 26 residues blocks exocytosis, the largest inhibitory C-terminal peptide must lie between these two extremes. Potentially, a larger peptide may inhibit secretion better than the 26-mer C-peptide (i.e. lower IC_{50}). C-terminal peptides shorter than 20-residues have not been examined.

It is likely that peptides homologous to parts of the first 80 N-terminal residues of SNAP-25 would also block exocytosis.

It will be appreciated that as the peptides shorten their inhibitory efficacies and avidities reduce notably ie. compare 20 and 26-mers (Ferrer-Mental *et al.*, 1998).

It is preferred that the said inhibitory SNARE may be capable of inhibiting exocytosis in a cell capable of performing SNARE-dependent exocytosis, (*in vitro* or *in vivo*), preferably a cell of the same or similar type to the said cell in a patient, by at least (in order of preference) 5, 10, 15, 20, 30, 40, 50, 60, 70, 80 or 90% compared to a control cell to which the said inhibitory SNARE is not supplied. Cell lines or cells which may be used *in vitro* to investigate the properties of an inhibitory SNARE may include adreno-chromaffin cells (see for example WO01/18038 and O'Sullivan *et al* (1999)), RIN (rat) and HIT (hamster) insulin-secreting cells (discussed in Gonelle-Gispert *et al* (1999)).

De Pavia *et al* (1999) *Proc Natl Acad Sci USA* **96**, 3200-3205 and Example 1 describe an *in vivo* system that may be used for assessing the effect of the inhibitory treatment described above and methods of reversal of inhibition. The method involves repeated *in vivo* imaging of nerve terminals and measurements of depolarisation-evoked endo- and exo-cytosis.

The inhibitory SNARE is preferably a fragment derivable by cleavage of synaptosomal-associated polypeptide of 25 kDa (SNAP-25) or a variant thereof by BoNT/A.

Still more preferably, the inhibitory SNARE consists of residues identical to residues 1 to 197 of full length SNAP-25 or a variant thereof ($SNAP\text{-}25_A$).

As discussed in WO01/18038, O'Sullivan *et al* (1999) and in Example 1, residues 1 to 197, 198, 199 or 200 of SNAP-25 inhibit exocytosis, whereas C-terminally longer forms, for example residues 1 to 202 of SNAP-25 are able to support exocytosis (and are therefore not inhibitory SNAREs).

BoNT/E-truncated SNAP-25 does not support exocytosis and significantly inhibits exocytosis (see Figure 8), but for a much shorter period than BoNT/A- or BoNT/C1-truncated SNAP-25. Its presence may promote intracellular movement and/or degradation of $SNAP\text{-}25_A$, as discussed in Example 1. Cleavage of a proportion of SNAP-25 or $SNAP\text{-}25_A$ molecules by BoNT/E therefore appears to be sufficient for the reversal of inhibition caused by the presence of $SNAP\text{-}25_A$. Supplying $SNAP\text{-}25_E$ molecules to a cell (for example by expression of the $SNAP\text{-}25_E$ in the cell) may also promote intracellular movement and/or degradation of $SNAP\text{-}25_A$ and may therefore also be useful in the reversal of inhibition caused by the presence of $SNAP\text{-}25_A$.

Suitable methods for detecting and identifying SNAP-25 and fragments derivable from SNAP-25 in cells are described in Example 1.

In a preferred embodiment the agent is capable of causing cleavage of the inhibitory SNARE. The product(s) of the cleavage preferably cause significantly less persistent inhibition than the uncleaved inhibitory SNARE. The agent may comprise a clostridial toxin, for example a botulinum toxin, by which the inhibitory SNARE is capable of being cleaved. In a particularly preferred embodiment the inhibitory SNARE is capable of being cleaved by BoNT/E and the agent comprises BoNT/E (or at least the catalytic portion ie light chain of BoNT/E). Thus, it is preferred in this embodiment that the inhibitory SNARE is not resistant to cleavage by BoNT/E ie is not a BoNT/E-resistant inhibitory SNARE. Replacement of the residue equivalent to I181 of full length human SNAP-25, for example by F, renders the variant resistant to BoNT/E; thus, it is preferred that the inhibitory SNARE is not an inhibitory SNAP-25 variant in which the

residue equivalent to I181 of full length human SNAP-25 is replaced, for example by F. Further examples of variants of SNAP-25 that are resistant to BoNT/E include variants in which the residue equivalent to residue 180 and/or the residue equivalent to residue 181 of full length SNAP-25 (for example full length mouse SNAP-25) are replaced by a residue other than Arg or a residue other than Ile, respectively. For example, Ile181 may be replaced by Phe, Gly, Ser or Asn. Replacement by Val may also result in a small increase in resistance to BoNT/E cleavage (Vaidyanathan *et al* (1999) *J Neurochem* 72, 327-337). Arg176, Asp179 and/or Met182 may further or alternatively be mutated, for example to Pro176, Lys179 and/or Thr182 (see Gonelle-Gispert *et al* (1999). Thus, such variants are not preferred.

By BoNT/E is included any variant, fragment, derivative or fusion of naturally occurring BoNT/E that retains the catalytic activity of BoNT/E, particularly the ability to cleave SNAP-25 in the same place as naturally occurring BoNT/E. It is preferred that the BoNT/E retains the cell-binding specificity of naturally occurring BoNT/E (ie may retain the heavy chain of naturally occuring BoNT/E), as well known to those skilled in the art, for example when treating a patient with BoNT/A or BoNT/C poisoning. When treating a patient in which exocytosis has been inhibited in a cell type to which botulinum toxins do not bind, for example by supply (for example expression) of an inhibitory SNARE to the cell, it may be desirable for the BoNT/E to be targeted to (and taken up by, or expressed inside) that cell type, using methods known to those skilled in the art: in this case, it is not necessary for the BoNT/E to retain the cell binding specificity of naturally occuring BoNT/E. Thus, the light chain of BoNT/E may be retained but not the heavy chain. It will be appreciated that BoNT/E may be expressed in cells as an alternative to delivering the actual polypeptide. Thus, the agent

may comprise a polynucleotide encoding and capable of expressing BoNT/E, as defined above, for example encoding at least the catalytic portion of BoNT/E. Suitable delivery vehicles are described in WO01/18038, for example adenoviral vectors with cholinergic-specific promoters may be used.

It will be appreciated that a toxin-resistant, for example BoNT/E-resistant, SNARE may typically be a non-naturally occurring SNARE, ie a synthetic, protease-resistant variant of a naturally occurring SNARE that is capable of being cleaved by the said clostridial toxin. However, a toxin-resistant SNARE may be a naturally occurring toxin-resistant SNARE. SNAP-23, for example is a naturally occurring toxin-resistant SNARE (at least in certain species); an inhibitory SNARE derivable therefrom is therefore not preferred when the active ingredient of the agent is BoNT/E.

By a toxin-resistant SNARE is included the meaning that the toxin-resistant SNARE is cleaved by the relevant clostridial toxin to a lesser extent than a SNARE that is cleaved by the said clostridial toxin (for example SNAP-25 for BoNT/A, BoNT/C or BoNT/E; synaptobrevin for BoNT/B, D, F or G and TeTx; syntaxin for BoNT/C). By "cleaved to a lesser extent" is included the meaning that at least about 1.2, 1.5, 2, 4, 5, 10, 20, 50, 100, 200, 500, 1000, 2000, 5000, 10000, 20000, 30000 or 40000 more of the said clostridial toxin is required to cleave 50% of the said toxin resistant SNARE than is required to cleave 50% of full length human SNAP-25 (for BoNT/A, BoNT/C or BoNT/E) or full length human synaptobrevin (for BoNT/B, D, F or G and TeTx) or full length human syntaxin 1 (for BoNT/C) under the same conditions, for example the conditions employed in the experiments summarised in Table 1 and described in Example 1 of WO01/18038.

Methods suitable for determining the amount of clostridial toxin required to cleave 50% of a polypeptide will be well known to those skilled in the art and are described, for example, in Gonelle-Gispert *et al* (1999), Vaidyanathan *et al* (1999).

In an alternative preferred embodiment, the agent which is capable of reducing the amount of the inhibitory SNARE in the cell and/or altering the location of the inhibitory SNARE in the cell may be SNAP-25_E or a polynucleotide encoding and capable of expressing SNAP-25_E. The presence of SNAP-25_E in the cell may promote removal and/or relocation of the inhibitory SNARE (for example SNAP-25_A or SNAP-25_{C1}) and may therefore be useful in reducing persistent inhibition of exocytosis in the cell.

Using SNAP-25_E or a polynucleotide encoding SNAP-25_E may provide advantages over using BoNT/E, because the former agents may be usable in a wider range of cell types than BoNT/E. In addition, the former agents may be easier to control in use and more reliable.

In relation to any method or use of the preceding aspects of the invention, it may be desirable to supply a SNARE that is capable of functioning in SNARE-dependent exocytosis (functional SNARE) to the patient, particularly to an affected cell of the patient, as described in WO01/18038. This may speed recovery of exocytosis in the cell. Thus, if exocytosis has been inhibited by the presence of an inhibitory SNAP-25 molecule, for example SNAP-25_A, as discussed above, then it may be desirable to supply full length SNAP-25 or a functional equivalent, for example SNAP-23, to the cell in addition to the agent. The supply of the, for example, full-length SNAP-25 may be by administering the full-length SNAP-25 to the cell or by

expressing the full-length SNAP-25 (SNAP-25wt) in the cell, for example from a recombinant polynucleotide.

When the inhibitory SNARE was formed in the cell as a result of exposure of the cell to a clostridial toxin, for example BoNT/A or BoNT/C1, it may be desirable for the SNARE that is capable of functioning in SNARE-dependent exocytosis that is supplied to the cell to be resistant to cleavage by the said clostridial toxin, for example BoNT/A or BoNT/C1. In a preferred embodiment, the said functional SNARE is also resistant to cleavage by the agent. Thus, in a particularly preferred embodiment, a patient or cell with BoNT/A poisoning and/or in which SNAP-25_A is present may be treated using BoNT/E as the agent (to cleave the SNAP-25_A to the non-inhibitory SNAP-25_E), and by administering or expressing SNAP-25 that is resistant to both BoNT/A and BoNT/E.

A SNARE which is capable of inhibiting the clostridial toxin (toxin-inhibitory SNARE), as discussed in WO01/18038 may also usefully be supplied to the cell as indicated above.

The toxin-resistant SNARE or toxin-inhibitory SNARE may be a variant, fragment, derivative or fusion of a naturally occurring SNARE with the required or preferred properties (for example in relation to their ability to support SNARE-dependent exocytosis) as discussed in WO01/18038.

By "variants" of a polypeptide, for example of SNAP-25, syntaxin 1 or synaptobrevin, we include insertions, deletions and substitutions, either conservative or non-conservative. In particular, we include variants of the polypeptide where such changes do not substantially alter the activity of the

said polypeptide, for example the ability of the SNAP-25, syntaxin 1 or synaptobrevin to participate in a ternary complex comprising SNAP-25, syntaxin 1 or synaptobrevin (or homologues thereof) which is capable of supporting exocytosis, for example as described in WO01/18038.

By "conservative substitutions" is intended combinations such as Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr.

By "residue equivalent to" a particular residue, for example the residue Arg198 of full-length SNAP-25, for example mouse or human SNAP-25, is included the meaning that the amino acid residue occupies a position in the secondary or three dimensional structure of a native polypeptide, for example a SNAP-25 homologue or variant, corresponding to the position occupied by the said particular residue, for example Arg198, in the native secondary or three dimensional structure of full-length SNAP-25. It will be appreciated that Arg198 of full-length SNAP-25 is located towards the C-terminus of the polypeptide.

The residue equivalent to a particular residue, for example the residue Arg198 of full-length SNAP-25, may be identified by alignment of the sequence of the polypeptide with that of full-length SNAP-25 in such a way as to maximise the match between the sequences. The alignment may be carried out by visual inspection and/or by the use of suitable computer programs, for example the GAP program of the University of Wisconsin Genetic Computing Group, which will also allow the percent identity of the polypeptides to be calculated. The Align program (Pearson (1994) in: Methods in Molecular Biology, Computer Analysis of Sequence Data, Part

II (Griffin, AM and Griffin, HG eds) pp 365-389, Humana Press, Clifton). Thus, residues identified in this manner are also "equivalent residues".

It will be appreciated that particularly in the case of truncated forms of SNAP-25 or in forms where simple replacements of amino acids have occurred it is facile to identify the "equivalent residue".

The sequence for human SNAP-25 is given in, for example Zhao *et al* (1994) *Gene* **145(2)**, 313-314. The sequences for mouse SNAP-25a and SNAP-25b isoforms are given in Bark (1993) *J Mol Biol* **233**, 67-76 and Bark & Wilson (1994) *Gene* **139**, 291-292.

The three-letter and one-letter amino acid code of the IUPAC-IUB Biochemical Nomenclature Commission is used herein. The sequence of polypeptides are given N-terminal to C-terminal as is conventional. In particular, Xaa represents any amino acid. It is preferred that the amino acids are L-amino acids; in particular it is preferred that the amino acid residues immediately flanking (such as those within 10 to 20 residues) of the clostridial toxin cleavage site consists of L-amino acid residues but they may be D-amino acid residues.

The patient may have botulism, in particular botulism caused by, or by a strain producing BoNT/A or BoNT/C1 (or less preferably BoNT/E, BoNT/D, BoNT/F or BoNT/G), preferably BoNT/A. Types of botulism and methods of diagnosing botulism are known to those skilled in the art and are summarised above. It will be appreciated that the patient may be an infant, for example an infant with the symptoms of a "floppy baby", that has been diagnosed as having botulism, as described above and, for example, in

Greve *et al* (1993) *Monatsschr Kinderheilkd* **141(1)**, 33-35; Midùra (1979) *Rev Infect Dis* **1(4)**, 652-655; Puig de Centorbi (1998) *Zentralbl Bakteriol* **287(1-2)**, 61-6; Pickett (1982) *Muscle Nerve* **5(9S)**, S26-27.

It will be appreciated that the method of diagnosing botulism preferably allows the type of botulinum toxin that is responsible for the poisoning to be determined. Thus, it will be appreciated that the method of treatment of the invention described above may further comprise the steps of determining the type of the said clostridial, for example botulinum, toxin from which the patient is suffering and of selecting an appropriate agent (in the appropriate aspect of the invention) for use in the treatment. Similarly, in the medicament–related use of the invention described above, the type of the clostridial toxin from which the patient is suffering from/poisoned by may be determined. Preferably, the patient is suffering from/poisoned by BoNT/A or BoNT/C1, most preferably BoNT/A.

Methods of determining the type of botulinum toxin affecting a patient are well known to those skilled in the art and include antibody and nucleic acid (for example, PCR) based assays. Toxin may be identified, for example, from stool specimens, as reviewed, for example, in Pickett (1982) *Muscle Nerve* **5(9S)**, S26-27 and Cherington (1998) *Muscle Nerve* **21(6)**, 701-710.

The patient may have been injected with BoNT/A or a molecule having the catalytic activity of BoNT/A, for example such a molecule as described in WO01/14570. BoNT/A is the toxin type generally utilised in treating neuromuscular conditions and is available commercially from several sources; for example from Porton Products Ltd, UK under the trade name

"Dysport"™, and from Allergan, Inc., Irvine, California under the trade name "BOTOX"™.

A further aspect of the invention provides a kit of parts comprising (1) means for determining the type of clostridial, for example botulinum, toxin from which a patient is suffering or means for determining that a patient is suffering from a particular type of clostridal, for example botulinum, toxin (preferably BoNT/A or BoNT/C1) and (2) an agent as defined in relation to previous aspects of the invention, for example comprising BoNT/E, or comprising SNAP-25$_E$ or a polynucleotide encoding SNAP-25$_E$.

A further aspect of the invention provides a kit of parts comprising (1) an agent as defined in relation to previous aspects of the invention, for example comprising BoNT/E, and (2) an inhibitor of the (or a) clostridial, for example botulinum, toxin from which the patient is suffering or the cell has been exposed to. The inhibitor may preferably inhibit BoNT/A or BoNT/C1, for example when the agent comprises BoNT/E. The inhibitor may be a toxin-inhibitory SNARE or recombinant polynucleotide capable of expressing said toxin-inhibitory SNARE, as described in WO01/18038. The kit may further comprise means for determining the type of clostridial, for example botulinum, toxin from which a patient is suffering or means for determining that a patient is suffering from a particular type of clostridal, for example botulinum, toxin, as described above.

A further aspect of the invention provides a kit of parts comprising an inhibitory SNARE or polynucleotide encoding an inhibitory SNARE, and an agent capable of cleaving the inhibitory SNARE, as defined in relation to

the fifth and sixth aspects of the invention. The kit thus provides means for inhibiting exocytosis, and means for reversing such inhibition.

In relation to all relevant aspects of the invention, it is preferred that the patient is a human. Less preferably, the patient may be a non-human mammal, for example a domesticated animal, for example a rodent (for example a mouse or a rat) or domesticated mammal, for example a horse or dog. It will be appreciated that many types of live stock or domesticated animals are susceptible to botulism. Further, it will be appreciated that the high level of sequence identity between equivalent SNAREs, for example SNAP-25s, from different animals, for example mammals, may mean that an inhibitory SNARE that is capable of inhibiting exocytosis in cells of one type of animal, for example mammal, may also be capable of inhibiting exocytosis in cells of a different type of animal and the methods/uses of the invention may therefore be useful in relation to treating both types of animal which have been treated using that inhibitory SNARE.

Typically, the patient to be treated is administered an effective amount of the said agent. By effective amount we include an amount sufficient to produce a clinically useful or significant reduction in any symptoms arising from the inhibition of exocytosis, for example symptoms of poisoning by a clostridial toxin, for example BoNT/A, in the said patient. The effective amount may produce an increase in exocytosis in the said cell. When the agent comprises BoNT/E, the time elapsed before clinically useful or significant reduction in any symptoms are apparent may depend on the dose of BoNT/E used, but may be within about 5 to 10 days. Generally, if more BoNT/E is used, the shorter the recovery time may be. A shorter recovery

time may be achieved if the agent comprises SNAP-25_E or a polynucleotide encoding SNAP-25_E rather than BoNT/E.

It will be appreciated that the methods or constructs of the invention may be evaluated in, for example, dissociated primary neuronal cell cultures, motor neurons, chromaffin cells and/or nerve-muscle co-cultures, as known to those skilled in the art, before evaluation in whole animals. The methods described in de Pavia *et al* (1999) *Proc Natl Acad Sci USA* **96**, 3200-3205 may also be used in the evaluation of the methods or constructs of the invention.

It will be appreciated that in treating a case of botulinum or tetanus poisoning, it may be beneficial to administer/deliver the delivery vehicle or genetic construct systemically; however, it may also or alternatively be beneficial to administer/deliver the delivery vehicle or genetic construct to the respiratory muscles (or other muscles showing paralysis) as a priority, for example by injection into the respiratory muscles, with or without more disseminated delivery.

In relation to reversing deliberate inhibition of exocytosis in particular cells, for example in reversing paralysis of particular muscles, it is desirable to deliver the BoNT/E (or other agent, as appropriate) as far as is practicable to the affected cells only. Thus, the BoNT/E (or other agent, as appropriate) may be injected at the site of the affected cells. Thus, the BoNT/E (or other agent) may be injected at the same site as the moiety that was used to produce the deliberate inhibition, or may otherwise be administered in the same manner.

The methods may be used in conjunction with other methods, for example administration of neutralising antibodies and/or BoNT inhibitors. The patient may be assessed in order to determine the stage of poisoning in order to decide on the most appropriate combination and/or order of treatment. Thus, the order may be administration of neutralising antibodies; administration of BoNT inhibitors; followed by rescue of regulated exocytosis by replacement with full length protein (as described in WO01/18038) and/or methods as described herein, for example supply of BoNT/E or SNAP-25_E.

Examples of suitable vectors and delivery systems, including systems in which expression of the encoded polypeptide is under the control of an inducible promoter, are described in, for example WO01/18038.

A further aspect of the invention provides the use of BoNT/E or a polynucleotide encoding and capable of expressing BoNT/E, or SNAP-25_E or a polynucleotide encoding and capable of expressing SNAP-25_E, in the manufacture of a medicament for the treatment of a patient in need of short duration inhibition of exocytosis in a cell of the patient, wherein the medicament does not comprise BoNT/A, B, C, F or G.

A further aspect of the invention provides a method for treating a patient in need of short duration inhibition of exocytosis in a cell of the patient, wherein the patient is administered BoNT/E and is not administered BoNT/A, B, C, F or G.

A further aspect of the invention provides a method for treating a patient in need of short duration inhibition of exocytosis in a cell of the patient,

wherein the patient is administered a recombinant polynucleotide encoding and capable of expressing BoNT/E; or SNAP-25$_E$; or a recombinant polynucleotide encoding and capable of expressing SNAP-25$_E$. It is preferred that the patient is not administered BoNT/A, B, C, F or G. It will be appreciated that it is desirable for expression of BoNT/E or SNAP-25$_E$ to be transient or to be capable of being controlled temporally, for example to be under the control of an inducible promoter, as well known to those skilled in the art and as discussed in WO01/18038. Preferably the inducer molecule may be suitable for oral administration, and is preferably administered in this way.

A further aspect of the invention provides a recombinant polynucleotide encoding and capable of expressing BoNT/E; SNAP-25$_E$; or a recombinant polynucleotide encoding and capable of expressing SNAP-25$_E$, for use in medicine.

A further aspect of the invention provides a pharmaceutical composition comprising a recombinant polynucleotide encoding and capable of expressing BoNT/E; SNAP-25$_E$; or a recombinant polynucleotide encoding and capable of expressing SNAP-25$_E$ and a pharmaceutically acceptable excipient. Suitable excipients and formulations will be well know to those skilled in the art and are described in, for example, WO01/18038, and may include sterile saline solution or distilled water which is pyrogen free.

A further aspect of the invention provides a gene therapy construct comprising a recombinant polynucleotide encoding and capable of expressing BoNT/E or a recombinant polynucleotide encoding and capable of expressing SNAP-25$_E$.

Examples of suitable vectors, gene therapy constructs and delivery systems which may be adapted in relation to the present invention, including systems in which expression of the encoded polypeptide is under the control of an inducible promoter, are described in, for example WO01/18038.

It was not previously known that BoNT/E differs from other botulinum toxins, for example BoNT/F, in that it does not elicit nerve sprouts and therefore is less likely than other botulinum toxins to cause permanent damage to the treated cells, as shown in Example 1. Use of BoNT/E (or the rapidly degraded SNAP-25$_E$) is therefore highly preferred in situations where short-term muscle weakness or immobilisation, followed by full recovery of muscle strength, is required.

The patient may be in need of inhibition of exocytosis of less than 14 days' duration, still more preferably in need of inhibition of exocytosis of less than 7, 6 or 5 days' duration.

The patient may be in need of inhibition of muscle contraction. Thus, the patient may be in need of temporary immobilisation of a joint or prevention of muscle contractions prior to, during or after surgery, treatment of joint dislocation, alleviation of muscle spasm, treatment of tendons or ligaments, treatment of scoliosis or spasm of sphincter muscles. The patient may be in need of relief of pain arising from muscle contractions.

Thus, the invention provides a method for inhibiting muscle contraction, relieving pain, temporarily immobilising a joint or preventing muscle contractions prior to, during or after surgery, treating joint dislocation,

alleviating muscle spasm, treating tendons or ligaments, treating scoliosis or spasm of sphincter muscles, wherein the patient is administered BoNT/E or a polynucleotide encoding and capable of expressing BoNT/E, or SNAP-25_E or a polynucleotide encoding and capable of expressing SNAP-25_E, and wherein the patient is in need of short duration of the effects of treatment and is not administered BoNT/A, B, C, F or G.

The invention further provides the use of BoNT/E or a polynucleotide encoding and capable of expressing BoNT/E, or SNAP-25_E or a polynucleotide encoding and capable of expressing SNAP-25_E in the manufacture of a medicament for inhibiting muscle contraction, relieving pain, temporarily immobilising a joint or preventing muscle contractions prior to, during or after surgery, treating joint dislocation, alleviating muscle spasm, treating tendons or ligaments, treating scoliosis or spasm of sphincter muscles, wherein the patient in need of short duration of the effects of treatment and is not administered BoNT/A, B, C, F or G.

As noted above, it is desirable for expression of BoNT/E or SNAP-25_E to be transient or to be capable of being controlled temporally, for example to be under the control of an inducible promoter, as well known to those skilled in the art and discussed in WO01/18038. It is preferred that the inducible promoter is controlled by an inducer molecule which is suitable for oral administration.

The patient may be suffering from a sports injury or muscle cramps. The patient may be suffering from a tension headache.

Smooth muscle disorders that may be treated include spasms of the sphincter of the cardiovascular arteriole, gastrointestinal system, urinary or gall bladder or rectum.

The patient may be undergoing or about to undergo total joint replacement, treatment of compound fractures, treatment of joint infections or dislocations.

It may be appropriate to treat a patient first with BoNT/E and then with BoNT/A in the same muscle group to provide a longer duration of action. This may be useful if, for example, recovery from an operation was delayed or took longer than anticipated.

The patient may alternatively be in need of short duration treatment of conditions such as cholinergic controlled secretions including excessive sweating, lacrimation, salivation and mucus secretions. This may be useful for a performer such as an actor, musician or public speaker during a performance or presentation.

Botulinum toxin E may exist in a dichain form or a single chain (un-nicked) form. The single chain form is less active than the dichain form but may be converted to the corresponding dichain form by nicking with a protease, for example trypsin. Both the single and the dichain form may be useful in relation to the present invention. An appropriate activatable recombinant neurotoxin as described in WO01/14570 may be used (ie one which has BoNT/E catalytic activity). By BoNT/E is included any variant, fragment, derivative or fusion of naturally occurring BoNT/E that retains the catalytic activity of BoNT/E, particularly the ability to cleave SNAP-25 in the same

place as naturally occurring BoNT/E. It is preferred that the BoNT/E retains the cell-binding specificity of naturally occuring BoNT/E, as well known to those skilled in the art. When the BoNT/E is expressed in the desired cell, it is not necessary for the cell-binding portion of the wt BoNT/E to be expressed.

Botulinum toxins may be obtained commercially or by establishing and growing cultures of appropriate *C. botulinum* strains in a fermenter and then harvesting and purifying the fermented mixture in accordance with known techniques. Commercial sources of BoNT/E are mentioned in Example 1.

Preferably the toxin (or in relation to the fifth and sixth aspects of the invention, other agent) is administered by means of intramuscular injection (when appropriate for the condition to be treated) directly into a local area such as a spastic muscle, preferably in the region of the neuromuscular junction, although alternative types of administration (for example subcutaneous injection), which can deliver the toxin directly to the affected region, may be employed where appropriate. The toxin may be presented as a sterile pyrogen-free aqueous solution or dispersion and as a sterile powder for reconstitution into a sterile solution or dispersion, as known to those skilled in the art.

Tonicity adjusting agents such as sodium chloride, glycerol and various sugars may be added, as known to those skilled in the art. Formulations suitable for use with other botulinum toxins, for example BoNT/A, as known to those skilled in the art, may be suitable for use with BoNT/E.

Suitable formulations may be described in, for example WO95/17904 and WO94/28923. Preferred unit dosage formulations are those containing a daily dose, daily sub-dose or an appropriate fraction thereof, of an active ingredient.

The dose of BoNT/E, SNAP-25$_E$ or polynucleotide (or where appropriate, other agent, as defined above) administered to the patient may depend upon the severity and extent of the botulinum toxin poisoning or inhibition of exocytosis to be treated. For example, it may depend on the number of muscle groups (or other cell types) requiring treatment, the age and size of the patient, and the type of toxin or inhibitory SNARE causing the poisoning/inhibition. Examples of useful doses and of methods of assessing useful doses are described in Example 1.

The potency of the toxin may be expressed as described in Example 1 (ie equivalent dose (ED) in relation to generation of maximal loss of toe spread reflex in mice without other obvious symptoms of botulism). Less preferably, the potency of the toxin may be expressed as a multiple of the LD_{50} value for the mouse, one unit (U) of toxin being defined as being the equivalent amoung of toxin that kills 50% of a group of 18 to 20 female Swiss-Webster mice, weighing about 20 grams each, within 4 days.

For example, a dose of between about 2 to 0.1, preferably 1 to 0.2, still more preferably between about 0.7 and 0.3 ED of BoNT/E may be useful in reversing paralysis of mouse muscle caused by about 0.5 ED of BoNT/A, measured as described in Example 1. Larger doses may be required in larger animals or target tissues.

The BoNT/E (or other agent) may be administered in a single or multiple doses. The quantity administered and the frequency of administration will be at the discretion of the responsible physician and will depend on the response of the patient to the treatment.

As well known to those skilled in the art, before injecting any muscle group with the BoNT/E, SNAP-25$_E$ or polynucleotide (or agent, where appropriate), the anatomy of the muscle group is considered carefully, the aim being to inject the area with the highest concentration of neuromuscular junctions, if known, or the area previously injected, for example with BoNT/A). Before injecting the muscle, the position of the needle in the muscle may be confirmed by putting the muscle through its range of motion and observing the resultant motion of the needle end. General anaesthesia, local anaesthesia and sedation are used according to the age of the patient, the number of sites to be injected, and the particular needs of the patient. More than one injection and/or sites of injection may be necessary in order to achieve the desired result. It may be necessary (depending on the required site of injection) to use a fine, hollow, Teflon™-coated needle, guided by electromyography. Suitable administration techniques are described in, for example WO95/17904 and WO94/28923.

The improvement in the patient's condition may be assessed subjectively and/or objectively.

The invention is now described by reference to the following, non-limiting, figures and examples.

Figure legends

Fig. 1: Time courses for recovery from neuromuscular paralysis induced by BoNT/E or F are faster than that seen following type A injection into mouse leg muscles: BoNT/E shortens the duration of the action of type A but not F toxin. (A) After intramuscular injection of one ED of BoNT/A (●), BoNT/E (O) or BoNT/F (▼) into the right hind-leg of mice, loss of neuromuscular transmission was assessed by determining the TSR score (5 is maximum paralysis). (B) Co-injection of 0.33 of an ED of BoNT/F with the same dose of BoNT/A (O), administration of the latter amounts of BoNT/E and BoNT/A (●), and co-application of 0.33 of an ED of type E and an equivalent dose of F (▼). Data presented are the means of 12 assays ± SD. (C) Sequential-injection of 0.63 (σ), 0.44 (■) and 0.31 (●) of an ED of BoNT/E, following a 3-day prior injection of 0.5 of an ED of BoNT/A.

Fig. 2: The extensive remodeling and switching in synaptic activity between the original nerve endings and their sprouts following paralysis with BoNT/A was less pronounced with BoNT/F poisoning and not detectable after type E. Using a Zeiss Axioskop microscope equipped with epifluorescence, injection of 0.05 ED of BoNT/A into mouse sternomastoid muscle was shown to result in a loss of the ability of the original endplates (stained with 4-di-2-ASP, green in A, filled bars in D) to exo-endocytose FM1-43 (red) upon stimulation with 60 mM K^+ (A and D) and an outgrowth of sprouts (arrows) capable of stimulated uptake of this dye (A and empty bars in D). Although a depletion of FM1-43 uptake in BoNT/E (0.05 ED) poisoned terminals was evident 1 day after injection (B and E), recovery of endo-/exocytosis occurred by d5 (E) and no sprouts were elicited. Within 1 day of injecting 0.05 ED of BoNT/F, FM1-43 uptake was dramatically reduced at the terminals (C and F); from d7, short sprouts

capable of stimulated FM1-43 endocytosis were seen and uptake into the original endings started to resume which was complete at d21, the time at which endocytotic activity of the sprouts began to subside (C and F). Bars = 20 μm. (Bars = 10 μm).

Fig. 3: Persistence of SNAP-25$_A$ in BoNT/A-treated murine motor nerve terminals. Control and BoNT/A-treated endplates were dual-labeled with rhodamine-conjugated α-bungarotoxin and anti-SNAP-25$_A$ antibody, followed by FITC-conjugated secondary IgGs; fluorescent images were recorded by confocal microscopy, as detailed in Materials and Methods. In control sternomastoid muscle, SNAP-25$_A$ could not be detected whereas positive immuno-staining was found on BoNT/A-treated preparations where it co-localized within areas occupied by the nAChR (d6-40), as revealed by the overlaid images. (Bars = 10 μm).

Fig. 4: Distribution of total SNAP-25 at the NMJ during BoNT/A-induced paralysis: disappearance of the sprouts following subsequent injection of BoNT/E. Control and BoNT/A-treated endplates were dual-labeled with rhodamine-conjugated α-bungarotoxin and anti-SNAP-25$_{FL}$ followed by FITC-conjugated secondary IgGs; the images were recorded by confocal microscopy. In the control, SNAP-25$_{FL}$ was detected in nerve terminals where it co-localized with areas occupied by the nAChR. In BoNT/A-treated preparations, some immunostaining was detected beyond the boundaries of nAChR, in sprouts (d6, d20; see arrows). Note that at day 20 after an injection of BoNT/E 3 days after BoNT/A, the sprouts were no longer detectable. (Bars = 10 μm).

Fig. 5: Rapid clearance of SNAP-25$_E$ from BoNT/E-treated motor nerve terminals. Control and BoNT/E-treated endplates were dual-labeled with rhodamine-conjugated α-bungarotoxin and anti-SNAP-25$_E$ followed by FITC-conjugated secondary IgGs. The fluorescent patterns were visualised by confocal microscopy. The latter immunostaining was apparent in nerve terminals and pre-terminal axons two days after BoNT/E injection but became undetectable by d7 post-injection. (Bars = 10 μm).

Fig. 6: Fate of SNAP-25$_A$ and SNAP25$_E$ at the NMJ upon sequential injection of BoNT/E 3 days after type A. Control and BoNT/A-treated mouse sternomastoid followed (after 3 days) in the latter case by injection of BoNT/E were dual-labeled with rhodamine-conjugated α-bungarotoxin and either anti-SNAP-25$_A$ or -SNAP25$_E$ followed by FITC-conjugated secondary IgGs. Confocal microscopy revealed that SNAP25$_A$ was detectable up to 11 days after BoNT/E injection in a few branches of the motor nerve terminals and pre-terminal axons; this staining was no longer seen 4 days later (d15). SNAP-25$_E$ was present at d5 but was not visible 11 days after BoNT/E injection. (Bars = 10 μm).

Fig. 7: Distribution and quantitation of SNAP-25$_{FL}$ and SNAP-25$_A$ immunostaining during BoNT/A treatment alone or with a subsequent injection of BoNT/E 7 days later. Nerve terminals in mouse sternomastoid were treated with BoNT/A alone (A, B) and additionally with BoNT/E 7 days later (C), as in Fig. 6, and then stained with IgGs specific for SNAP-25$_{FL}$ (A) or SNAP-25$_A$ (B, C); all the samples were labeled with rhodamine-conjugated α-bungarotoxin, followed by FITC-conjugated secondary antibodies. After visualisation by confocal microscopy, the fluorescent intensity profiles from representative cross-sections (white lines)

were recorded and quantified, using Image Pro Plus V4, for 22-55 endplates per condition from at least 2 preparations. The localisation of SNAP-25_{FL} (A) and SNAP-25_A (B) at the plasma membrane in BoNT/A-treated endplates was revealed (D, E) by the close apposition of their fluorescence intensity peaks (green lines) with that for membrane-bound nAChR (red lines). A contrasting pattern was seen for samples treated with BoNT/A and /E (7 days later) in that SNAP-25_A was no longer in apposition with the nAChR labeling; instead, the SNAP-25_A staining had shifted to between the 2 peaks of AChR on opposing membranes, suggestive of movement to the cytosol (F). (Bars = 10 μm).

Figure 8: BoNT/A or E truncated SNAP-25, shown to be expressed in CHO cells, inhibited evoked secretion in intact chromaffin cells. (A) CHO cells, that lack SNAP-25, were transfected with the pcDNA1.1/Amp vector incorporating the specified SNAP-25-R198T gene using Superfect™ reagent, as described in O'Sullivan *et al* 1999. A membrane fraction was isolated from the cells and equal amounts of the SDS-solubilised proteins were subjected to SDS-PAGE and immunoblotting, using the indicated antibodies. (b) Intact chromaffin cells were transfected with the pcDNA1.1/Amp mammalian expression vector containing the SNAP-25, SNAP-25_A or SNAP-25_E together with the reporter plasmid encoding hGH, as outlined in O'Sullivan *et al* (1999). After five days, evoked Ba^{2+}-induced hGH secretion (means ± S.E.M.; n=3) was obtained as expressed as a percentage of the measured total cell hGH content. Data are representative of two separate experiments. ** represent unpaired t-test results, comparing the extent of SNAP-25_E inhibition to that of SNAP-25_A, which was significant. SNAP-25(1-197) is SNAP-25_A; SNAP-25(1-180) is SNAP-25_E.

Example 1: Recovery of synaptic activity to mouse endplates paralysed by botulinum toxin type A is hastened by the short-acting type E toxin due to the removal of truncated SNAP-25

Quantal neurotransmitter release is inhibited selectively by seven serotypes (A-G) of botulinum neurotoxin (BoNT) whose Zn^{2+}-dependent protease cleaves SNARE proteins that are essential for this fundamental process of Ca^{2+}-regulated exocytosis. Although BoNT/A and /E proteolyse SNAP-25 at neighboring bonds, their blockade of acetylcholine release from mouse motor nerves following local injection caused flaccid muscle paralysis for very different durations (30 and 5 days, respectively). By *in vivo* imaging of synaptic vesicle endo-/exocytosis at individually-identified nerve terminals, BoNT/E injection into mouse muscle was shown to inhibit depolarisation-dependent uptake of the dye, FM1-43, but the vesicle recycling resumed after 5 days and there was an absence of detectable nerve sprouting. In contrast, neuroparalysis resulting from BoNT/A or /F induced the appearance of nerve sprouts, that exhibited FM1-43 uptake, and these were eliminated when the parental terminals recovered functionality. In fact, the extent and life-time of the sprouts are related reciprocally to the duration of neuromuscular paralysis by the various toxins. Immunostaining and confocal microscopy revealed a transient presence of BoNT/E-truncated SNAP-25, and a contrasting persistence of the BoNT/A-cleaved target at the nerve plasmalemma for up to 40 days. The product of BoNT/A, SNAP-25_A, appears to be a hindrance to rehabilitation of the original terminals because co-injection of BoNT/E hastened its removal and the subsequent recovery of neurotransmission.

The extended neuroparalysis produced by BoNT/A elicits nerve sprouting and the synapses created by these sprouts seems to be responsible for the initial synaptic activity produced on the onset of recovery from muscular paralysis (de Paiva *et al.*, 1999). The idea that removal of muscle activity by denervation provides the stimulus for sprouting was first established by Lomo (see review (Lomo, 1976)). Elimination of these outgrowths depends on muscle activity since direct electrical stimulation prevented or reversed sprouting (Brown *et al.*, 1981; Lomo, 1976). Upon blockade of regulated exocytosis by BoNT/A, numerous studies have indicated that sprouting continues well after the initial resurgence of nerve-induced muscle twitch (Angaut-Petit *et al.*, 1990; de Paiva *et al.*, 1999; Juzans *et al.*, 1996; Molgó *et al.*, 1990). Indeed, it was recently established that the return of activity to the originally-poisoned nerve endings coincides with the onset of sprout elimination (de Paiva *et al.*, 1999). Since sprouts are not as efficient in mediating exo-endocytosis as the parent terminal, it was suggested that once a threshold level of activity is reached at the original terminal, the muscle responds by signaling sprout elimination (de Paiva *et al.*, 1999).

In this study, the latter proposal was confirmed and extended with the use of other BoNT serotypes, alone or in conjunction, to manipulate the extent and time course of the sprouting process. Moreover, the much longer-lasting paralysis induced by BoNT/A compared to the rapid, transient effect of BoNT/E was found to be due, at least in part, to the persistence of BoNT/A-truncated SNAP-25 ($SNAP\text{-}25_A$) in the plasmalemma where it could prevent neurotransmitter release. This defective protein was found to be translocated from the presynaptic membrane and removed from the terminal following co-injection of the other SNAP-25-targeting toxin, BoNT/E; such dis-inhibition of the trafficking of the $SNAP\text{-}25_A$ would allow

replenishment of the intact active protein and could overcome the proteolytic action of any BoNT/A activity remaining.

Abbreviations: botulinum neurotoxin/type A, E, BoNT/A, E; effective dose, ED; nicotinic acetylcholine receptors, nAChRs; cleaved products of BoNT/A and /E, SNAP-25_A and SNAP-25_E; PBS, phosphate-buffered saline; TSR, toe spread reflex; 4-di-2-ASP, 4-(4-diethyl aminostyryl)-N-methylpyridinium iodide; FM1-43, N-(3-triethyl ammonium propyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide.

Methods

Comparison of recovery times from neuromuscular paralysis induced by BoNT/A, /E and /F using digital abduction scoring in mice. Tyler's ordinary mice (20 g) were lightly anaesthetized with halothane (4%) and injected (0.1 ml) intramuscularly into the dorsal surface of the right hind leg with an effective dose (ED) of either BoNT/A-haemaglutinin complex [BOTOX (List Biologics Ltd., California), 5 pg], pure BoNT/E supplied by Dr. B.S. DasGupta (50 pg) or BoNT/F complex (4 ng)], or dual combinations [BoNT/A/E 5 pg and 25 pg, respectively, or /A/F 5 pg and 2 ng, respectively)]. BoNT/A, E and F are also available from other sources, for example Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset, BH12 4QH, UK (catalogue numbers B8776, B6528 and B9152 respectively). It should be noted that the ED determined for each toxin gave maximal paralysis of the *extensor digitorus longus* muscle in the absence of any other symptoms of botulism in the mice. The mice were allowed to recover and the loss of toe spread reflex (TSR) scored from 0 to 5 (where 5 is a complete

absence of TSR) following their examination twice daily (Pockett and Gavin, 1985).

Repeated visualization of endplates on the sternomastoid muscle in mice. Surgical procedures and repeated visualization of nerve terminals before and after injection of BoNT/A, E or F were performed, as detailed elsewhere (de Paiva *et al.*, 1999). Briefly, Tyler's ordinary mice (15-20g) of either sex were anaesthetized with fentanyl citrate 0.079 mg/mL / fluanisone 2.5 mg/mL / midazolam 0.5 mg/mL (10 mL/kg, i.p.); the right sternomastoid muscle was exposed and lifted onto a flattened wire. Nerve endings were stained for 5 min with either 5 μM 4-(4-diethyl aminostyryl)-N-methylpyridinium iodide (4-di-2-ASP; Molecular Probes) alone in aerated Krebs-Ringer medium ([mM] NaCl, 118; KCl, 4.69; $MgSO_4$, 1.18; KH_2PO_4, 1.18; glucose, 11.7; $NaHCO_3$, 23.8; $CaCl_2$, 2.52, pH 7.4; (de Paiva *et al.*, 1999), or in Krebs-Ringer with elevated K^+ concentration (60 mM KCl and 58 mM NaCl) containing both 5 μM 4-di-2-ASP and 4 μM N-(3-triethyl ammonium propyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide (FM1-43; Molecular Probes). In both instances, the wound was then extensively washed with normal Krebs-Ringer over 3 min. After placing a circular coverslip mounted on a second wire over the endplate region, each mouse was positioned under a Zeiss Axioskop fixed-stage microscope equipped with epifluorescence. Staining with 4-di-2-ASP was visualized with a FITC-type narrow band-pass filter block (450-490 nm excitation λ, 515-565 nm emission λ) with detection of labeling with 4-di-2-ASP and FM1-43 being achieved using the above filter and a long-pass rhodamine-type block (524-556 nm excitation λ, ≥ 590 nm emission λ), respectively. In order to prevent tissue photo damage, the excitation intensity was minimized and images recorded with an intensified CCD video camera (Jai 758; Datacell, UK) and Image Pro Plus v3.0 (Datacell) imaging software. The same single terminals were repeatedly

observed (de Paiva *et al.*, 1999; Lichtman *et al.*, 1987) for up to 6 weeks (in the case of BoNT/A) through Zeiss 20x/0.50 and 40x/0.75 water immersion objectives. Following acquisition of the initial images (d0 time point), BoNT/A-haemagglutinin complex, BoNT/E or BoNT/F complex (all at 0.05 ED in 2μL/20g body weight) was injected into the sternomastoid muscle within 2 mm of the endplate region. The incision was closed with 4-5 sutures. Toxin-treated nerve endings were later visualized for a second time by repeating the above procedures. Stored images of activity-dependent uptake of FM1-43 and staining with 4-di-2-ASP at the nerve terminals and their outgrowths, were pseudo-coloured (red and green, respectively) and overlaid without modification of their luminance values; then the latter levels in each colour channel were quantified by calculating the average intensity values for a band of lines encompassing the entire sprout or terminal with Image Pro Plus v3.0. Intensity integrations and statistical analysis were performed after exporting the data to Excel 97. Sprout length was also quantified using Image Pro Plus.

Immunolabeling and laser scanning confocal microscopy. Control, BoNT/A- and/or E- poisoned sternomastoid muscles were processed as in de Paiva *et al.*, 1999. Antibody staining was carried out using a 1:100 dilution of the primary antibodies raised in rabbits against recombinant glutathione-*S*-transferase SNAP-25, synthetic peptides corresponding to residues 190-197 (anti-SNAP-25_A) or 173-180 (anti-SNAP-25_E) of mouse SNAP-25. The latter two antibodies have been shown to display absolute specificity for their respective immunogens (Sesardic *et al.*, 1994; Lawrence *et al* (1997); O'Sullivan *et al* (1999)). For localizing endplates, postsynaptic nicotinic acetylcholine receptors (nAChRs) were dual-stained with rhodamine-conjugated α-bungarotoxin. Fibres were then washed in PBS and mounted with Fluoprep (BioMerieux, Marcy l'étoile, France). Samples were imaged with a laser scanning microscope (Zeiss 510)

mounted on an upright microscope (Axioplan-2 Zeiss) and operated with the manufacturer's software (LSM 510 version 1.49.44) running on Windows NT 4.0 operating system (Microsoft, U.S.A.). The 488 and 543 nm lines of an Argon-ion and Helium-Neon-ion laser, respectively, were used for excitation with intensity minimized to 4% of 25 mW and 30 % of 30 mW power, respectively. Images were collected using an oil-immersion objective (plan-Neofluar, 60x/1.4) and separated with a combination of a FITC-type narrow band-pass filter block (505-530 nm) and a long-pass rhodamine-type block (≥ 560 nm). Images were analyzed using Image Pro Plus (V4; Datacell). The mean densities of fluorescence per pre-terminal axon, nerve terminal, and sprouts were measured for the various immunolabelled antigens, at different times after intoxication with BoNT/A, /E or both.

Results

Recovery from BoNT/A poisoning in mice is slower than for BoNT/E or /F: neuroparalyis times are inversely proportional to the duration and extents of the nerve sprouting induced.

To establish the time course of the recovery following an injection of these toxin serotypes into the hind-leg of mice, equivalent doses were first established for BoNT/A, /E and /F which caused a maximal loss of toe spread reflex (TSR, score = 5) without other obvious symptoms of botulism; this was termed the effective dose (ED). In the case of BoNT/A, such complete paralysis was seen within 48h of the injection and sustained for a further 3-4 days before a gradual protracted resumption of TSR commenced, with full function being regained only after 28-30 days (Fig. 1A). Upon injecting one ED of BoNT/E, which like type-A cleaves SNAP-25 albeit at a different site, gave a complete loss of TSR within 24 h (Fig. 1A). In contrast to type A, however, recovery from BoNT/E-induced paralysis was extremely rapid with normal TSR being observed within

4.5 days (Fig. 1A). For comparison, BoNT/F which cleaves the vesicle protein – synaptobrevin – was tested. A total absence of TSR was observed 36 – 48h after injecting one ED and sustained for less than 24 h; indeed, within 7-8 days, a complete recovery of muscle function was obvious (Fig. 1A).

Repeated viewing of the same identified endplates in living mice (de Paiva *et al.*, 1999) was utilized to examine the sprouting response to the shorter-acting BoNT/E or /F compared to BoNT/A which has an extended duration of effectiveness. This allowed monitoring of the ability of the original motor nerve endings and any nascent sprouts to undergo stimulated uptake of the fluorescent exo-endocytotic marker, FM1-43. Imaging of nerve terminals on the sternomastoid muscle, immediately prior to injection of BoNT/A (d0) revealed co-localization of the green dye, 4-di-2-ASP, which labels nerve endings, and activity-dependent staining with red FM1-43 giving a yellow fluorescence (Fig. 2A). The latter was diminished 2 days following intoxication (data not shown, see de Paiva *et al.*, 1999) and an extensive network of sprouts capable of stimulated uptake of this dye appeared 2-5 days thereafter, reaching a maximum length of 150.6 μm after 42 days (Table 1). Upon the return of nerve-stimulated muscle twitch at day 28, regulated vesicle recycling occurred solely in the sprouts (Fig. 2A and D) and not at the parent terminals, indicating that only these sprouts can be responsible for this initial phase of the recovery of nerve-muscle transmission. Injection of BoNT/E also resulted in a rapid diminution of FM1-43 uptake (Fig. 2B and E) so that within 24 h levels equivalent to that of non-stimulated nerve endings (data not shown) were evident. In contrast to BoNT/A poisoned terminals, recovery of synaptic activity in the original commenced within 48h of administering BoNT/E and no sprouts could be detected (Table 1); indeed, by day 5 when nerve-induced muscle contraction had recovered (Fig. 1A), FM1-43 uptake was equivalent to that measured before intoxication (Fig.

2B and E). When BoNT/F was injected into the sternomastoid muscle, stimulated FM1-43 uptake into the nerve endings was diminished within 24-48 h (Fig. 2C and F) but, unlike BoNT/E, short sprouts were formed by day 7. These immediately acquired the capability of stimulated exo-endocytosis but, in contrast to type A, FM1-43 uptake levels at the original endplate began to recover within 7 days (Fig. 2F), at which time nerve-induced muscle twitch had also returned (Fig. 1A). These outgrowths reached a maximum length and complexity after 14 days (Table 1, Fig. 2C) and, thereafter, started to retract (Fig. 2C) so that by day 21-27 the endplates resembled those seen before intoxication.

Together, these data indicate that signaling for sprouting requires a loss of exo-endocytosis at the original endplates for between 3 and 5 days; if rehabilitation of regulated exo-endocytosis at the original endplate occurs prior or within this period, no sprouts are elicited. Also, it is reasonable to deduce that the sprouting is switched off when activity returns to the original endplates, although it may take 2-3 days for this process to manifest itself.

Molecular basis of the different recovery times for BoNT/A- or /E-induced paralysis at the neuromuscular junction. Nerve terminals of sternomastoid muscle were stained throughout BoNT/A-induced paralysis with an antibody reactive exclusively with the C-terminus of SNAP-25_A and the patterns compared with the those seen with IgGs raised against full-length SNAP-25. Additionally, control and BoNT/A-injected samples were counter-stained with rhodamine-conjugated α-bungarotoxin to locate post-synaptic nAChR and, thereby, pinpoint the endplates. As expected, no SNAP-25_A was detected in untreated terminals (Fig. 3) but 6 days post-injection this product could be detected pre-synaptically within the area demarkated by nAChR; no staining could be detected within the sprouts. 20 days after BoNT/A treatment an intense

staining of the nerve terminals with SNAP-25_A IgG was observed which persisted up to d 40, albeit at a diminished intensity. However, by d 50 SNAP-25_A could no longer be detected within the BoNT/A-treated nerve terminals.

These collective findings demonstrate that BoNT/A-cleaved SNAP-25 persists in the original endplates and this is clearly related to the prolonged neuroparalysis because only when this product disappears (day 50) does exo-endocytosis resumes therein. Since BoNT/E-induced paralysis is of such a limited duration (Fig. 1A), it was pertinent to investigate the turnover of SNAP-25_E. This was done using an antibody only reactive with the C-terminus of SNAP-25_E. Indeed, the very short-lasting effect of BoNT/E was substantiated by the transient appearance of SNAP-25_E immunoreactivity; it was only detectable 2 days after treatment and had disappeared by day 7 (Fig.5).

Is complete recovery from BoNT-induced neuromuscular paralysis a function of substrate turnover or toxin persistence? A fundamental question remained to be answered: why, in the case of BoNT/A poisoning are the original endplates unable to undergo neurotransmitter release for such a prolonged period whereas the other SNAP-25-targetted toxin, serotype E, causes a contrasting short-lived paralysis? To investigate this, the action of BoNT/E was studied in conjunction with BoNT/A. If the durations of the paralysis were solely dependent on the lifetime of the neurotoxins within the nerve terminals, then co-administering both serotypes should give a recovery profile dependent on the longer-lasting BoNT/A. Injection of BoNT/A and /E together into the hind-leg of mice revealed that type E significantly shortens the recovery time from BoNT/A poisoning from ~28 days to 12.5 days (Fig. 1B). This seems to indicate that active BoNT/A is not at an adequate level within the nerve terminals to maintain an extended paralysis under those conditions (see later) and supports

the hypothesis made previously (Eleopra *et al.*, 1998; Washbourne *et al.*, 1998) that the rapid recovery from BoNT/E poisoning may be attributable to its particular cleavage site on SNAP-25. Accordingly, staining of EDL with anti-SNAP-25 IgG 20 days after exposure to BoNT/A, with and without BoNT/E, showed that no sprouts could be detected in the dual-injected samples whilst treatment with BoNT/A above showed extended sprouting, as expected (Fig. 4). An alternative possibility is that co-injection could reduce BoNT/A uptake into the terminals if BoNT/E acts faster and impairs the activity-dependent internalisation of BoNT/A that is known to occur at motor nerve terminals (Black and Dolly 1986; Simpson, 1980) and make the extracellular BoNT/A more susceptible to proteolysis. To test this hypothesis, BoNT/E (0.5 of the ED) was injected 3 days after an initial administration of BoNT/A (0.5 of its ED); this should allow adequate time for BoNT/A to establish its paralysis pattern. A full recovery from this procedure was observed at ~ day 13 after the second injection (Fig. 1C), corresponding to the time course of recovery following the co-injection (Fig. 1B). It is, therefore, unlikely that the recovery from the sequential or co-injection of BoNT/E with BoNT/A is due to an impaired uptake of the latter. On the contrary, it indicates BoNT/E speeds up the molecular events underlying the recovery of nerve-induced muscle twitch (Eleopra *et al.*, 1998). This was substantiated by the demonstration that varying the BoNT/E dose administered following the initial injection of BoNT/A altered the recovery time accordingly (Fig. 1C). Further to this, injection of BoNT/F together with BoNT/A did not alter extensively the time to recovery of the TSR (Fig. 1B). This indicates that, as BoNT/F is targeted to synaptobrevin, the persistence of BoNT/A-cleaved SNAP-25 is preventing the faster recovery of neurotransmitter release shown to occur following treatment with BoNT/F alone (Fig. 1A).

In view of the observed ability of BoNT/E to dramatically shorten the long-lasting paralysis triggered by BoNT/A, it was pertinent to check whether

the further cleavage of SNAP-25_A by BoNT/E could result in more rapid clearance from the motor nerve terminal, thereby allowing the recovery process to proceed. Thus, the sternomastoid muscle was first injected with BoNT/A and the mice were re-operated 3 days later and BoNT/E administered. At various times thereafter, the samples were probed with antibodies specific for SNAP-25_A and SNAP-25_E. Fig. 6 presents evidence that the SNAP-25_A produced was present 11 days after BoNT/E injection but this staining was virtually undetectable at later time points [d 15, Fig.6 and d26 (data not shown)]. Surprisingly, on the same neuromuscular preparation, SNAP-25_E was detected at d 5 after BoNT/E injection, but was absent at day 11 (Fig.6). The observed longer persistence of SNAP-25_A suggests that not all of it had been subject to further cleavage by BoNT/E, or SNAP-25_A continued to be produced by any remaining active BoNT/A. Analysis of immunoreactivity levels and the relative distributions of SNAP-25_{FL} (Fig. 7A) and SNAP-25_A (Fig. 7B) in control and BoNT/A-treated endplates showed that the majority of these two products resided in the neuronal plasma-membrane, as reflected by their staining being in close proximity to nAChR located on the muscle (Fig. 7D, E). In BoNT/A – treated terminals exposed 7 days later to BoNT/E, the distribution of SNAP-25_A (Fig. 7C) was found to be more cytosolic than membrane-bound (Fig. 7F). This suggests that as a consequence of the later treatment with BoNT/E, SNAP-25_A is transported in membrane vesicles with SNAP-25_E to a more cytosolic location. Taken in conjunction with the evidence presented above for BoNT/E accelerating the removal of SNAP-25_A, it may reasonably be deduced that this represents the first step in the clearance of SNAP-25_A from these nerve terminals.

Discussion

The abolition of neurotransmitter release from motor-nerve endings by injecting a sublethal dose of BoNT/A complex elicits the appearance of exocytosis-competent nerve sprouts within 3-5 days (de Paiva *et al.*, 1999). The latter is reminiscent of the quantal-like stimulated release measured from neuronal extensions of cultured motoneurons (analogous to nerve sprouts) by whole-cell patch-clamp recording from myocytes making contact along the processes (Zakharenko *et al.*, 1999). A requirement for the sprouts in the recovery from BoNT/A paralysis has been hypothesized previously (Brown *et al.*, 1981) but the ultimate link became apparent with the demonstration that at the onset of nerve-induced muscle twitch at day 28-30, sprouts are the sole synaptic structures capable of undergoing exo-endocytosis (de Paiva *et al.*, 1999). This represents the first phase of recovery with the second coinciding with the restoration of exo-endocytosis at the original nerve endings. In this context, the data presented herein on various BoNT serotypes suggest that the initiation and maintenance of the sprouts relate to the duration of inactivity of the original endplates. We show that the initiation of sprouting requires the poisoned terminals to be devoid of exo-endocytotic activity for more than 3-5 days because BoNT/E failed to elicit nerve outgrowths and gave only a transient (~5 days) blockade of neuromuscular transmission; apparently, sprouting can be avoided by a rapid restoration of synaptic activity at the nerve terminals. Accordingly, BoNT/F did elicit moderate endplate remodeling and caused a slightly more prolonged paralysis (~7 days).

Although it is known that the sprouts are ultimately eliminated with concomitant return of activity to the parent endplate (Hassan *et al.*, 1994), a more detailed understanding of this elimination process has only recently been possible (de Paiva *et al.*, 1999). It had previously been thought that the sprouts would stop growing once nerve-induced muscle twitch recovered, this deduction being based on the prevention of BoNT/A-induced sprouting by chronic electrical

stimulation of the soleus muscle (Brown *et al.*, 1980). But several studies have failed to support this dogma since sprout elongation continues well after the onset of the first phase, characterised by the initial restoration of muscle twitch (Angaut-Petit *et al.*, 1990; Holds *et al.*, 1990; Juzans *et al.*, 1996; Molgó *et al.*, 1990). Recently, convincing evidence was presented that the trigger for sprout elimination is not this first phase but is, in fact, the rehabilitation of the originally-poisoned motor nerve terminals (de Paiva *et al.*, 1999). Moreover, it is shown herein that the time point for sprout elimination can be manipulated by over-riding the prolonged paralysis existence of SNAP-25_A in BoNT/A-paralysed preparations with a delayed injection of another SNAP-25-targetting serotype, BoNT/E, thereby, resulting in a shortening of the recovery process and, consequently, an earlier induction of sprout elimination (see Fig. 4 and 5). This leads directly to the other facet of this study, aimed at gaining a better understanding of the molecular basis for such a long inhibition by BoNT/A of neurotransmitter release at the original endplates. Such a phenomenon is even more striking in light of the rapid turnover of SNAP-25 in the optic tract and superior colliculi (Loewy *et al.*, 1991) and cultured PC-12 cells (Lane and Liu, 1997). The long-lasting blockade of release by BoNT/A could only be affected by: (i) an extended life-time of BoNT/A protease activity within the motor neurons (Keller *et al.*, 1999) and neuro-endocrine cells (O'Sullivan *et al.*, 1999) and/or (ii) impairment of SNAP-25_{FL} incorporation at the release sites due to persistence of SNAP-25_A (one possibility mentioned by Eleopra *et al.*, 1998). Co-injection of BoNT/A and /E was shown to shorten the paralysis time expected for type-A intoxication, which seems to preclude persistence of adequate activity of the BoNT/A toxin within the original endplate (Eleopra *et al.*, 1998). Indeed, if BoNT/A-proteolytic activity was chronically persistent when compared to BoNT/E, a much longer paralysis should have been reported. In sharp contrast, the lifetime of BoNT/A was found to exceed by far that of

BoNT/E in a spinal cord neuronal culture treated sequentially with 0.4 pM BoNT/A followed 3 days later with 250 pM (Keller *et al.*, 1999). One of the explanation put forward to explain such a discrepancy was that during the co-injection protocol, a faster poisoning by BoNT/E could have somehow prevented binding and internalisation of BoNT/A. Although the two toxins do not compete for the ecto-acceptors at the NMJ (Dolly, 1990; Dolly *et al.*, 1994), which by itself rules out this possibility, sequential injection of the two toxins was performed to allow BoNT/A to achieve a full paralysis before applying BoNT/E. In accordance with the results of reported co-injection experiments (Eleopra *et al.*, 1998), the later injection of BoNT/E greatly shortened the recovery expected for BoNT/A paralysis. Therefore, SNAP-25_A seems to be a major hindrance to recovery of neurotransmitter at the original endplates and this, probably, results from a competition between SNAP-25_A and SNAP-25_{FL} for SNARE binding partners at release sites. In this view, it is intriguing that BoNT/E, which target the same substrate, elicits a much shorter recovery. Could it be that SNAP-25_A and SNAP-25_E have different turnover rates, as previously proposed (amongst other possibilities; Eleopra *et al.*, 1998)? And if so why? Various levels of SNAP-25_A were detected at the original motor nerve terminals by immunocytochemistry from day 3 to 40 following injection of BoNT/A. By day 50 such staining was no longer apparent which correlates with the time frame for the initiation of rehabilitation of original endplate (de Paiva *et al.*, 1999). In comparison, BoNT/E-poisoned original endplates were shown to be devoid of SNAP-25_E immunolabelling by day 7, confirming that both products are dealt with differentially. Sequential injection of BoNT/E following a prior injection of BoNT/A was shown to hasten the clearance of SNAP-25_A and correlated with an acceleration of the recovery from paralysis. Although SNAP-25_E immunoreactivity had disappeared from the terminals 12 days after BoNT/E treatment following BoNT/A, some SNAP-25_A staining was still detectable

albeit exhibiting a pattern distinct from that for SNAP-25_{FL} or SNAP-25_A in terminals treated with either /A or /E alone. Apparently, after sequential injection of BoNT/A and BoNT/E, SNAP-25_A was translocated from the pre-synaptic plasma membrane to a more central location, probably cytoplasmic. These results also revealed that BoNT/E did not target all the SNAP-25_A even though the latter is a substrate for the toxin (Lawrence *et al.*, 1997). Still, BoNT/E did initiate a series of reactions culminating in the removal of SNAP-25_A most probably by endocytosis and retrograde transportation. Hence, it is proposed that SNAP-25_A can enter a SNARE complex, thereby rendering it exocytosis-incompetent and, furthermore, inaccessible to newly-synthesised SNAP-25. In this context, (Huang *et al.*, 1998) have observed that BoNT/A treatment or SNAP-25_A over-expression increases the number of docked vesicles in HIT-T15 insulinoma cells. Removal of residues from the C-terminus of SNAP-25 does not affect its binary interaction with syntaxin but slightly reduces it with VAMP (Hayashi *et al.*, 1994; Hayashi *et al.*, 1995; Chapman *et al.*, 1994; Raciborska and Charlton, 1999; Canaves and Montal, 1998). The formation of the ternary complex is reduced (50%) but not prevented. In contrast, further cleavage of 17 residues by BoNT/E totally inhibits the formation of the ternary complex (Hayashi *et al.*, 1995). Interestingly, (Banerjee *et al.*, 1996) have shown in permeabilised PC-12 cells that BoNT/E completely blocks Ca^{2+}-activated exocytosis of large dense core vesicles whereas the BoNT/A-induced inhibition was only partial. Moreover, it has been reported that the subsequent addition of BoNT/E after BoNT/A drastically decreases this BoNT/A-insensitive exocytosis in permeabilised chromaffin cells (Lawrence *et al.*, 1997). Thus, SNAP-25_E is incapable of entering this SNARE complex, leaving it unprotected and exposed to clearance mechanisms operating within motor nerve terminals. This is further supported by the findings of Raciborska *et al* (1998) in which the persistence of SNAP-25_A at the plasma membrane was

demonstrated in frog NMJ; this population of truncated target could only be cleaved from the plasma membrane following the concomitant removal of syntaxin.

To explain the duration of exo-endocytotic blockade resulting from BoNT/A action, the scenario must be different. We propose that following BoNT/A entry into nerve terminals, two types of ternary complexes co-exist: one with SNAP-25_{FL} (protected from cleavage) and the other with SNAP-25_{A}. Both complexes are capable of supporting exocytosis on application of a strong stimulus (Banerjee *et al.*, 1996; Lawrence *et al.*, 1997), with the former being far more efficient. In that view, simultaneous detection of vesicle fusion and catecholamine by capacitance measurement and amperometry has allowed 2 bursts of catecholamine exocytosis to be distinguished; both were abolished by BoNT/E but only the slower event was diminished by BoNT/A in chromaffin cells (Neher, 1998). The complex formation could explain why it is not eliminated at release sites and replaced by SNAP-25_{FL}, thereby accounting for the prolonged inhibition. The constitutive pathway is known to be responsible for delivery of newly synthesised SNAP-25 through a cycle of exocytosis (Gonzalo *et al.*, 1999; Kelly *et al.*, 1993; O'Sullivan *et al.*, 1999) and for replacement of defective SNAP-25 from the plasma membrane through vesicle-mediated recycling (Walch-Solimena *et al.*, 1995). Our results suggest that only SNARE-embedded SNAP-25 is involved in exo-endocytosis and that it is differentially turned-over with regard to other forms of SNAP-25. Under physiological conditions, SNAP-25_{FL} is used by regulated exocytosis to operate in conjunction with the other SNAREs. Since SNAP-25_{E} cannot assemble into the ternary SNARE complex, it is therefore expelled from the plasma membrane by constitutive endocytosis coupled with retrograde transport and replaced by newly-synthesised SNAP-25. By contrast, SNAP-25_{A} enters the ternary SNARE

complex and is integrated into the regulated pathway competing with $SNAP25_{FL}$ for release sites. It is suggested that $SNAP\text{-}25_A$ could inhibit passage from the regulated exo-endocytotic pathway to retrieval of material by constitutive endocytosis and retrograde transport. Further experiments are needed to test this hypothesis which should be addressed with a more suitable model than NMJ in the hope of tackling the difficult question regarding the fate of the truncated products, and bringing clarity to such a complex series of events.

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CLAIMS

1. A method for treating a patient with Botulinum toxin A (BoNT/A) or Botulinum toxin C1 (BoNT/C1) poisoning, wherein the patient is administered Botulinum toxin E (BoNT/E) or a polynucleotide encoding and capable of expressing BoNT/E, or a fragment derivable by cleavage of synaptosomal-associated polypeptide of 25 kDa (SNAP-25) or a variant thereof by BoNT/E ($SNAP\text{-}25_E$) or a polynucleotide encoding and capable of expressing $SNAP\text{-}25_E$.

2. Use of BoNT/E or a polynucleotide encoding and capable of expressing BoNT/E, or $SNAP\text{-}25_E$ or a polynucleotide encoding and capable of expressing $SNAP\text{-}25_E$, in the manufacture of a medicament for the treatment of a patient with BoNT/A or BoNT/C1 poisoning.

3. A method for treating a patient in need of reversal of inhibition of exocytosis in a cell of the patient caused by contact of BoNT/A or BoNT/C1 with the said cell, wherein the patient is administered BoNT/E or a polynucleotide encoding and capable of expressing BoNT/E, or $SNAP\text{-}25_E$ or a polynucleotide encoding and capable of expressing $SNAP\text{-}25_E$.

4. Use of BoNT/E or a polynucleotide encoding and capable of expressing BoNT/E, or $SNAP\text{-}25_E$ or a polynucleotide encoding and capable of expressing $SNAP\text{-}25_E$, in the manufacture of a medicament for the treatment of a patient in need of reversal of inhibition of exocytosis in a cell of the patient caused by contact of BoNT/A or BoNT/C1 with the said cell.

5. Use of an agent which is capable of (1) reducing the amount of a fragment, variant, fusion or derivative of a SNARE or a fusion of a said fragment, variant or derivative (inhibitory SNARE) that is capable of inhibiting SNARE-dependent exocytosis in a cell in which an inhibitory SNARE is present, and/or (2) altering the location of the inhibitory SNARE in a cell in which an inhibitory SNARE is present, in the manufacture of a medicament for the treatment of a patient in need of reversal of inhibition of SNARE-dependent exocytosis in a cell in which the inhibitory SNARE is present.

6. A method for reversing the inhibition of SNARE (soluble (*N*-ethylmaleimide-sensitive fusion protein)-attachment protein receptor)-dependent exocytosis in a cell in which a fragment, variant, fusion or derivative of a SNARE or a fusion of a said fragment, variant or derivative (inhibitory SNARE) that is capable of inhibiting SNARE-dependent exocytosis is present, the method comprising the step of supplying to the cell an agent which is capable of reducing the amount of the inhibitory SNARE in the cell and/or altering the location of the inhibitory SNARE in the cell, wherein the method is performed *in vivo*.

7. A method for reversing the inhibition of SNARE (soluble (*N*-ethylmaleimide-sensitive fusion protein)-attachment protein receptor)-dependent exocytosis in a cell in which a fragment, variant, fusion or derivative of a SNARE or a fusion of a said fragment, variant or derivative (inhibitory SNARE) that is capable of inhibiting SNARE-dependent exocytosis is present, the method comprising the step of supplying to the cell an agent which is capable of reducing the amount of the inhibitory SNARE in the cell and/or altering the location of the inhibitory SNARE in

the cell, wherein the inhibitory SNARE is not present in the cell as a result of exposure of the cell to BoNT/A.

8. The method or use of claim 5 or 6 wherein the inhibitory SNARE is present in the cell as a result of circumstances other than exposure of the cell to BoNT/A.

9. The method or use of any of claims 5 to 8 wherein the inhibitory SNARE is present in the cell as a result of expression of the inhibitory SNARE in the cell or as a result of administration of the inhibitory SNARE to the cell.

10. The method or use of any one of claims 5 to 9 wherein the inhibitory SNARE is a fragment derivable by cleavage of synaptosomal-associated polypeptide of 25 kDa (SNAP-25) or a variant thereof by BoNT/A.

11. The method or use of any one of claims 5 to 10 wherein the said inhibitory SNARE consists of residues identical to residues 1 to 197 of full length SNAP-25 or a variant thereof ($SNAP\text{-}25_A$).

12. The method or use of any one of claims 5 to 11 wherein the agent is capable of causing cleavage of the inhibitory SNARE.

13. The method or use of any one of claims 5 to 12 wherein the agent comprises Botulinum toxin E or the catalytic portion of Botulinum toxin E or a polynucleotide encoding and capable of expressing BoNT/E or the catalytic portion of Botulinum toxin E.

14. The method or use of any one of claims 5 to 11 wherein the agent comprises SNAP-25$_E$ or a polynucleotide encoding and capable of expressing SNAP-25$_E$.

15. The method or use of any one of the preceding claims wherein the cell is a nerve cell, adreno-chromaffin cell or insulin-secreting cell.

16. The method or use of any of claims 1 to 6, 10 to 15 wherein the patient has botulism acquired naturally or accidentally.

17. The method or use of any of claims 1 to 6, 10 to 15 wherein the patient has been injected with BoNT/A or BoNT/C1 for medical purposes.

18. The method of any of the preceding claims wherein the patient is an infant.

19. A kit of parts comprising (1) means for determining the type of clostridial, for example botulinum, toxin from which a patient is suffering or means for determining that a patient is suffering from a particular type of clostridal, for example botulinum, toxin (preferably BoNT/A or BoNT/C1) and (2) BoNT/E or an agent as defined in any one of claims 5 to 14.

20. A kit of parts comprising (1) BoNT/E or an agent as defined in any one of claims 5 to 14 and (2) an inhibitor of a clostridial, for example botulinum, toxin, preferably BoNT/A or BoNT/C1.

21. A kit of parts comprising an inhibitory SNARE or polynucleotide encoding an inhibitory SNARE and an agent as defined in any one of claims 5 to 14.

22. Use of BoNT/E or a polynucleotide encoding and capable of expressing BoNT/E, or SNAP-25$_E$ or a polynucleotide encoding and capable of expressing SNAP-25$_E$, in the manufacture of a medicament for the treatment of a patient in need of short duration inhibition of exocytosis in a cell of the patient, wherein the medicament does not comprise BoNT/A, B, C, F or G.

23. A method for treating a patient in need of short duration inhibition of exocytosis in a cell of the patient, wherein the patient is administered BoNT/E or a polynucleotide encoding and capable of expressing BoNT/E, or SNAP-25$_E$ or a polynucleotide encoding and capable of expressing SNAP-25$_E$, and is not administered BoNT/A, B, C, F or G.

24. The use of claim 22 or method of claim 23 wherein the patient is in need of inhibition of exocytosis of less than 14 days' duration.

25. The use or method of claim 23 wherein the patient is in need of inhibition of exocytosis of less than 7, 6 or 5 days' duration.

26. The use or method of any one of claims 22 to 23 wherein the patient is in need of inhibition of muscle contraction.

27. The use or method of claim 26 wherein the patient is in need of temporary immobilisation of a joint or prevention of muscle contractions

prior to, during or after surgery, treatment of joint dislocation, alleviation of muscle spasm, relief of pain, treatment of tendons or ligaments, treatment of scoliosis or spasm of sphincter muscles.

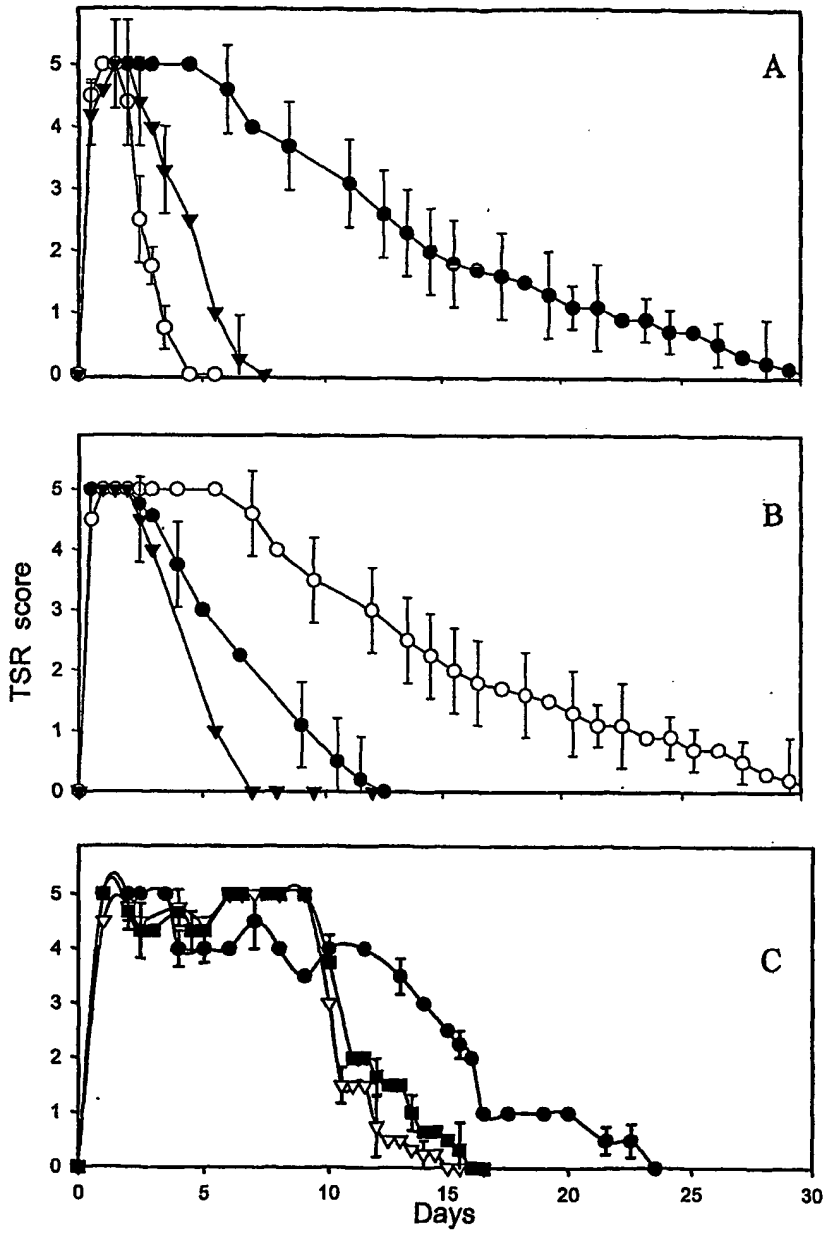
28. A recombinant polynucleotide encoding and capable of expressing BoNT/E; SNAP-25_E; or a recombinant polynucleotide encoding and capable of expressing SNAP-25_E, for use in medicine.

29. A pharmaceutical composition comprising a recombinant polynucleotide encoding and capable of expressing BoNT/E; SNAP-25_E; or a polynucleotide encoding and capable of expressing SNAP-25_E and a pharmaceutically acceptable excipient.

30. A gene therapy construct comprising a recombinant polynucleotide encoding and capable of expressing BoNT/E or a recombinant polynucleotide encoding and capable of expressing SNAP-25_E.

31. Any novel method of treatment, use, polypeptide, molecule, pharmaceutical composition or nucleic acid as herein disclosed.

Figure 1



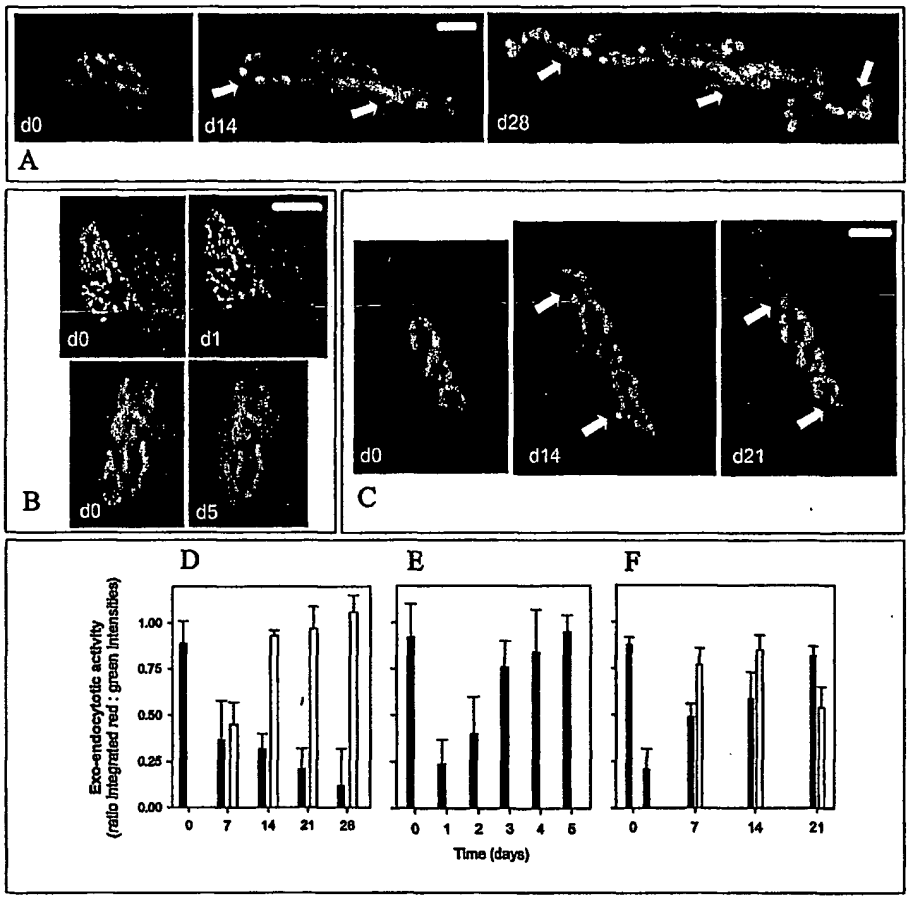

d0
d14
d28
A
d0
d1
d0
d5
B
d0
d14
d21
C
D
E
F
Exo-endocytotic activity
(ratio integrated red : green intensities)
1.00
0.75
0.50
0.25
0.00
0 7 14 21 28
0 1 2 3 4 5
0 7 14 21
Time (days)

Figure 2

Figure 3

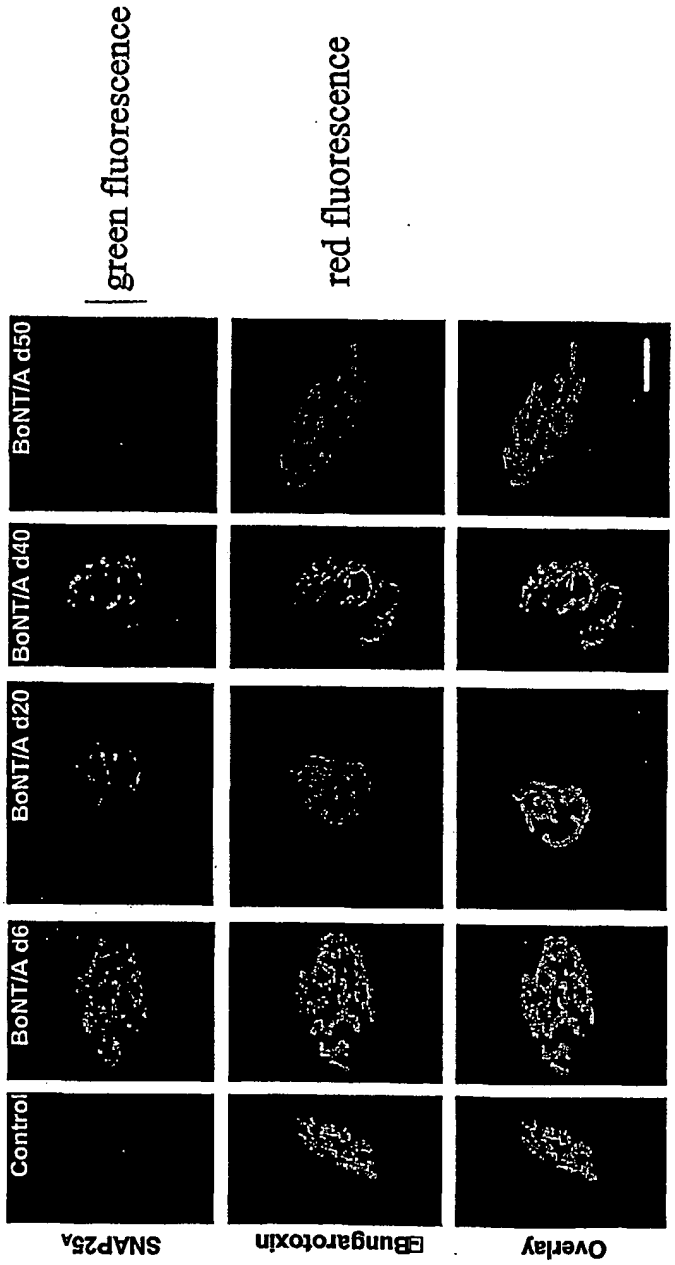

green fluorescence
red fluorescence
BoNT/A d50
BoNT/A d40
BoNT/A d20
BoNT/A d6
Control
SNAP25A
Bungarotoxin
Overlay

Figure 4

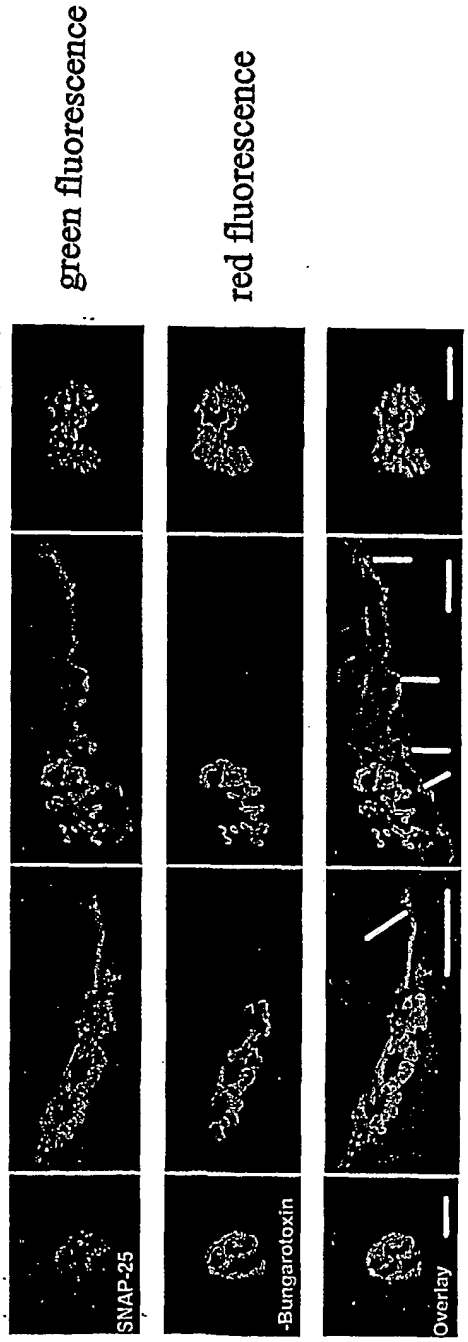
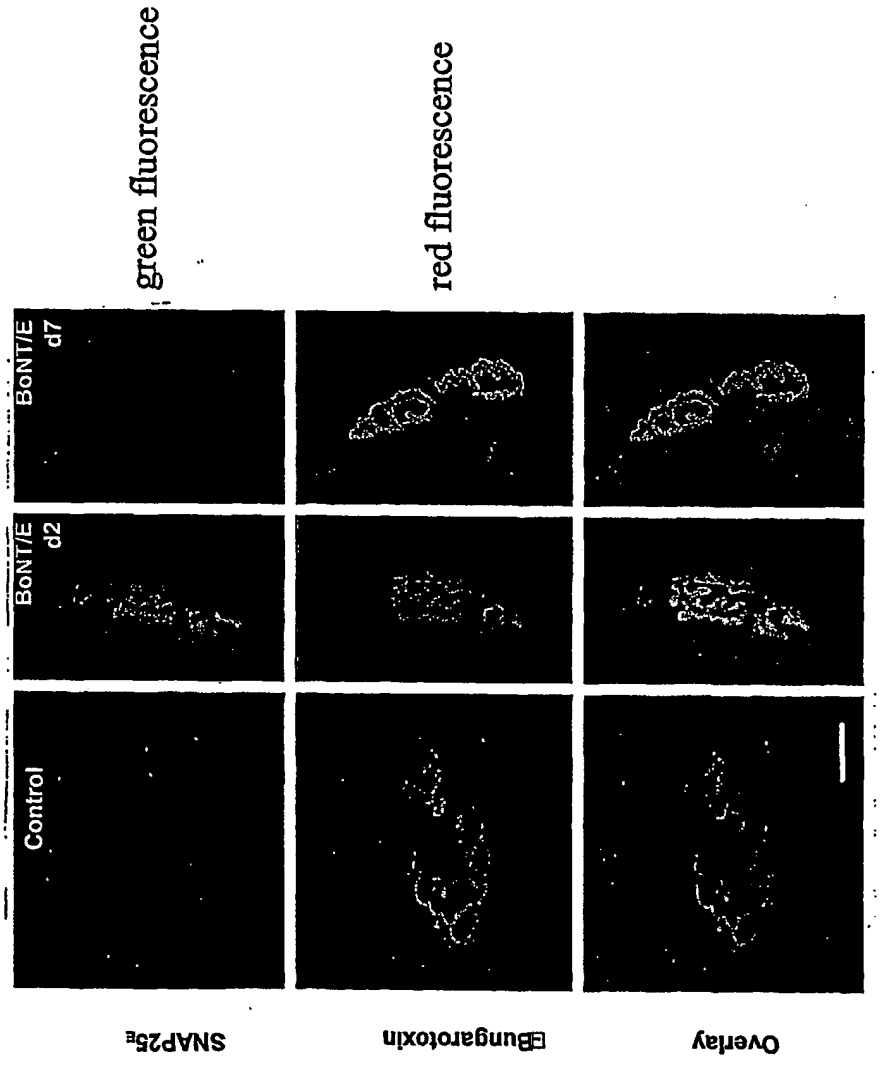

green fluorescence
red fluorescence
Control
BoNT/A d6
BoNT/A d20
BoNT/A and E d20
SNAP-25
-Bungarotoxin
Overlay

Figure 5


green fluorescence
red fluorescence
Control
BoNT/E d2
BoNT/E d7
SNAP25e
Bungarotoxin
Overlay

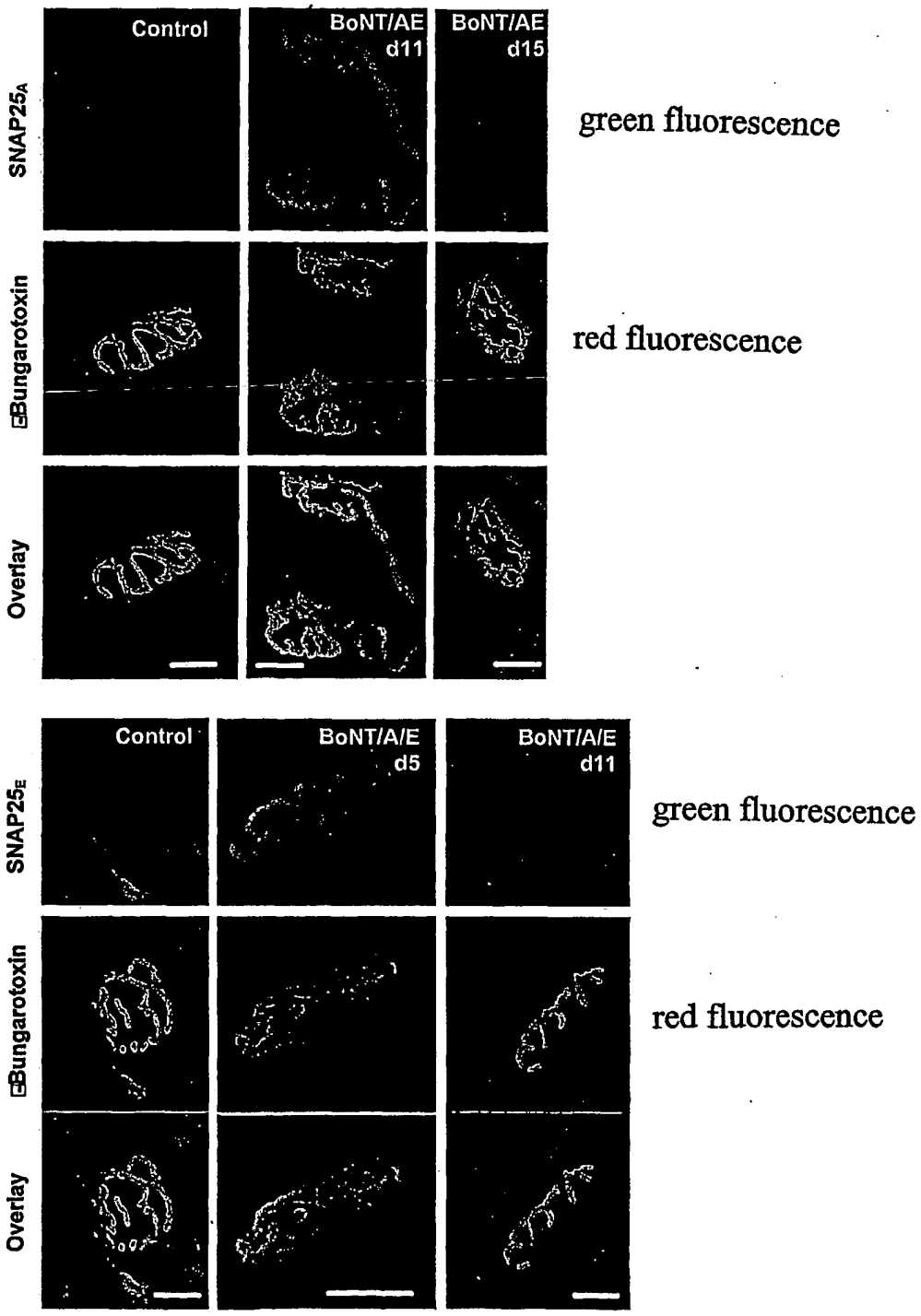

Control
BoNT/AE d11
BoNT/AE d15
SNAP25A
Bungarotoxin
Overlay
green fluorescence
red fluorescence
Control
BoNT/A/E d5
BoNT/A/E d11
SNAP25E
Bungarotoxin
Overlay
green fluorescence
red fluorescence

Figure 6

Figure 7

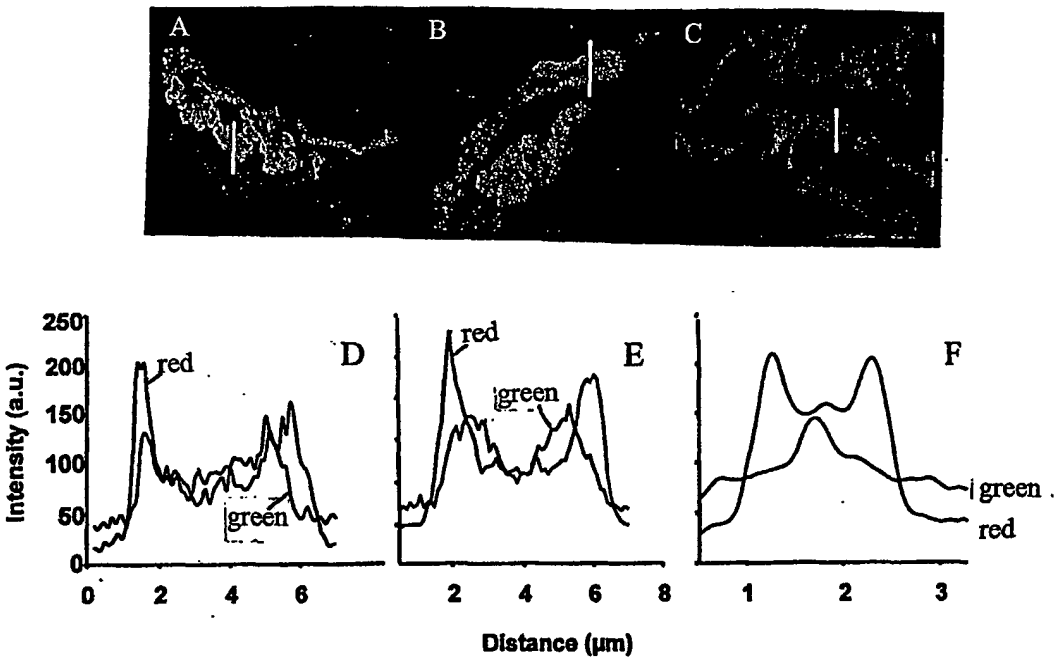
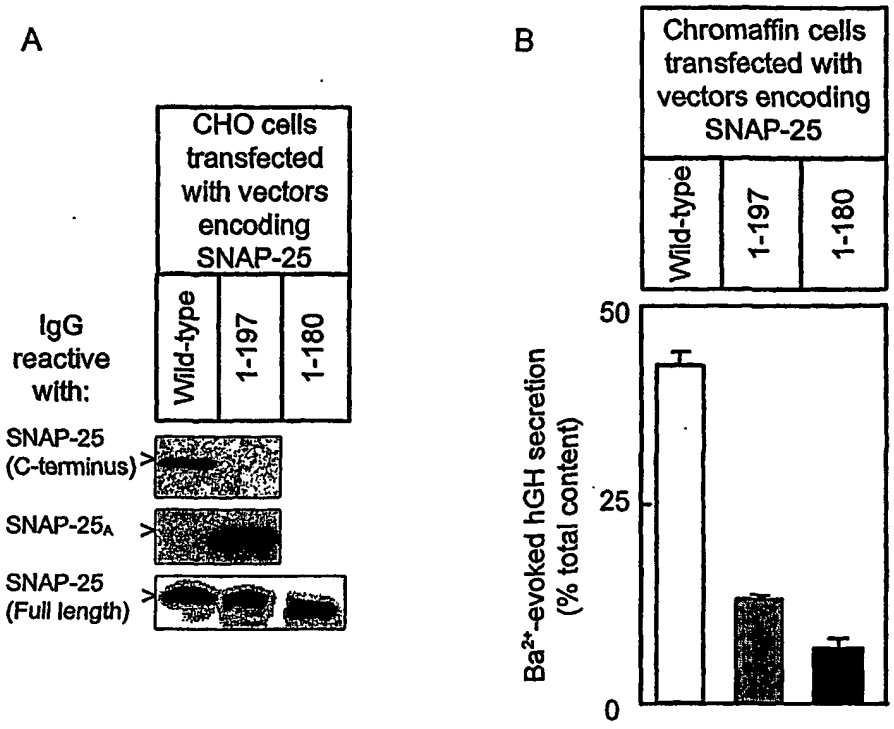

A
B
C
D
E
F
red
green
Intensity (a.u.)
250
200
150
100
50
0
0
2
4
6
8
1
3
Distance (µm)

Figure 8


A
CHO cells transfected with vectors encoding SNAP-25
Wild-type
1-197
1-180
IgG reactive with:
SNAP-25 (C-terminus)
SNAP-25A
SNAP-25 (Full length)
B
Chromaffin cells transfected with vectors encoding SNAP-25
Wild-type
1-197
1-180
Ba2+-evoked hGH secretion (% total content)
50
25
0

INTERNATIONAL SEARCH REPORT

Int...tional Application No
PCT/GB 02/02087

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/48 A61K38/17 A61K39/08 A61P21/02 A61P31/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, MEDLINE, CHEM ABS Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 17904 A (ALLERGAN INC) 6 July 1995 (1995-07-06) cited in the application page 4, line 29 -page 5, line 6 examples	22-27,31
X	US 6 214 602 B1 (ZDANOVSKY ALEXEY G) 10 April 2001 (2001-04-10) column 8, line 51-57 column 12, line 43-51 column 13, line 47; example 2	22-29,31
X Y	WO 97 34620 A (UNIV CALIFORNIA) 25 September 1997 (1997-09-25) page 2, line 6 -page 3, line 17 page 19, line 21 -page 20, line 10; claim 2	22-25 26-29

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 August 2002	03/09/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Deck, A

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 02/02087

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01 14570 A (CHAN KUO CHION ;DOLLY J OLIVER (GB); LI YAN (GB); ALLERGAN SALES I) 1 March 2001 (2001-03-01) page 10, line 20-22; examples 9-17	28-30
Y	PELLIZZARI ROSSELLA ET AL: "Tetanus and botulinum neurotoxins: Mechanism of action and therapeutic uses." PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY OF LONDON B BIOLOGICAL, vol. 354, no. 1381, 28 February 1999 (1999-02-28), pages 259-268, XP001098768 Feb. 28, 1999 ISSN: 0962-8436 page 264, left-hand column	26,27
Y	ELEOPRA ROBERTO ET AL: "Different time courses of recovery after poisoning with botulinum neurotoxin serotypes A and E in humans." NEUROSCIENCE LETTERS, vol. 256, no. 3, 13 November 1998 (1998-11-13), pages 135-138, XP001095571 ISSN: 0304-3940	28,29
A	page 137, right-hand column	1-21
Y	POULET S ET AL: "SEQUENCES OF THE BOTULINAL NEIROTOXIN E DERIVED FORM CLOSTRIDIUM BOTULINUM TYPE E(STRAIN BELUGA) AND CLOSTRIDIUM BUTYRICUM(STRAIN ATCC 43181 AND ATCC 43755)" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, ACADEMIC PRESS INC. ORLANDO, FL, US, vol. 1, no. 183, 28 February 1992 (1992-02-28), pages 107-113, XP001080566 ISSN: 0006-291X the whole document	28
A	ADLER MICHAEL ET AL: "Persistence of botulinum neurotoxin A demonstrated by sequential administration of serotypes A and E in rat EDL muscle." TOXICON, vol. 39, no. 2-3, February 2001 (2001-02), pages 233-243, XP001095570 ISSN: 0041-0101 the whole document	1-21

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 02/02087

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 01 18038 A (IMP COLLEGE INNOVATIONS LTD ;FORAN PATRICK G (GB); MOHAMMED NADIEM) 15 March 2001 (2001-03-15) the whole document	1-21

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 02/02087

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1, 3, 6-18, 23-27, 31 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 5-12,19-21 (partially)
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 5-12,19-21 (partially)

Present claims 5-12, 19-21 relate to the use of a compound defined by reference to a desirable characteristic or property, namely its ability to reduce the amount or alter the location of an ihnibitory SNARE. The claims cover all compounds having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT for only a very limited number of such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compound/ by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the compounds of claims 12 and 13.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 02/02087

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INTERNATIONAL SEARCH REPORT

Information on patent family members

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PCT/GB 02/02087

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